Molecular Diagnostics for Peanut Allergy

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12.1 The Peanut's Role as an Allergen

Peanut belongs to the family of legumes and is the most common cause of food-induced anaphylactic reactions. Being responible for the largest percentage of deaths among food allergens (Pumphrey 2000), peanuts are the most important primary food allergen. Following peanut provocations, respiratory difficulties are common (Ahrens et al. 2012). The stable nature of peanut allergens, as well as the relatively high proportion of total protein amount, contributes to the enormous health threat peanuts can pose. Peanut contains a high protein percentage of 24–29 % (Koppelman et al. 2001),

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mostly storage proteins, which leads to the low threshold for reactions in peanut allergy sufferers. Even extremely small peanut quantities (1.6 mg peanut protein) cause allergic reactions in 5 % of peanut-allergic individuals (Blom et al. 2013).

Epidemiology
In the USA and Great Britain, between 1 and 2 % of infants and young children have been diagnosed with a peanut allergy (Nicolaou and Custovic 2011), and in Australia the percentage lies at 3 %. In Germany, peanut allergy seems to be slightly less common. Nevertheless, 10.6 % of German children and teenagers have an elevated peanut-specific IgE (Schmitz et al. 2013). A multicenter and multinational study concerning the prevalence of sensitizations to food allergens among adults in Europe (EuroPrevall) showed a high variability (Burney et al. 2014). Using extract-based diagnostics, the sensitization rates varied between 0.5 % in Reykjavik, 5 % in Zürich, 1.6 % in Utrecht, and 7.2 % in Madrid. The analysis of the prevalence of peanut storage proteins sensitization rates, which is typical of childhood peanut allergy (Ballmer-Weber et al. 2015), significantly altered the picture: no sensitizations were recorded in Sofia and Lodz, 0.1 % in Utrecht, 0.4 % in Zürich, and 0.5 % in Madrid.

The high sensitization rates to peanut extract in different parts of Europe are caused by cross-reactions through:

- Birch Bet v t-homolog PR-10-proteins (Ara h 8)
- Lipid transfer proteins (Ara h 9) for patients in the Mediterranean region
- Profilins (Ara h 5)
- Carbohydrate determinant-(CCD-) carrying glycoproteins for patients with primary sensitizations to birch pollen (PR-10-proteins), peach-LTP (Pru p 3), or grass pollen (profilins and CCDs)

Peanut's Role in the Food Industry
In Europe and North America, peanuts are mostly consumed roasted, e.g., still in their shell, salted and peeled, or processed into peanut butter or peanut puffs. As a nonrefined product, peanut oil may contain relevant quantities of the peanut allergen and may cause allergic reactions. In Asian regions, raw peanuts are mostly consumed as an ingredient in cooked dishes. The allergenicity of raw peanuts decreases through a long cooking process. In contrast, roasting at very high temperatures facilitates the formation of compact, globular protein aggregates, which can augment the allergenicity of Ara h 1 and 2 (Vissers et al. 2011).

12.2 Individual Peanut Allergens

The clinical reactions are determined by the characteristics of the individual proteins (◉ Figs. 12.1 and 12.2, ◉ Table 12.1), especially when the sensitization encompasses a single allergen family. In addition, primary and secondary

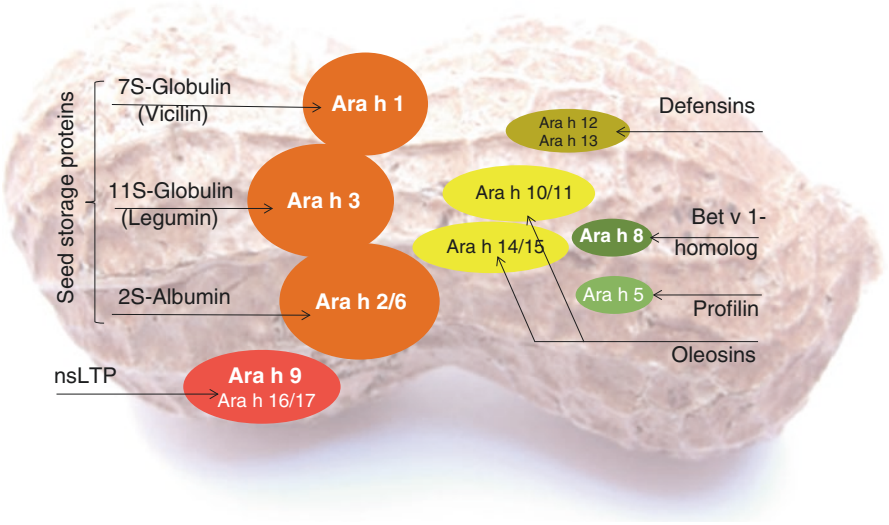

Fig. 12.1 Currently identified peanut allergens. The *ellipse* sizes roughly indicate their percentage of the total protein (***Fettdruck***: available for specific IgE diagnostic)

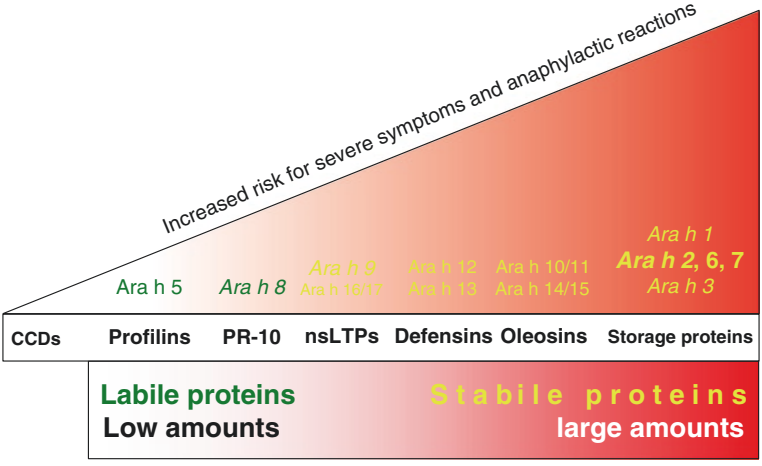

Fig. 12.2 Peanut allergens and their role in determining clinical symptoms according to the "risk ramp". While allergens which are unstable and occur in smaller quantities (left) tend to induce no or mild oropharyngeal symptoms, IgE sensitizations to those which are stable and occur abundantly (right) are more commonly associated with severe allergic symptoms

allergens are differentiated: the primary (class I) food allergens induce sensitization via the cutaneous or gastrointestinal route, whereas the secondary (class II) food allergens mainly cross-react to structurally similar epitopes, e.g., following predominantly an inhalant sensitization.

Table 12.1 Peanut allergens (www.allergen.org, 03-04-2016)

Allergen	Biochemical name	MW	Heat stability
Ara h 1	Cupin (vicillin-type, 7S globulin)	64	Yes
Ara h 2	Conglutin (2S albumin)	17	Yes
Ara h 3	Cupin (legumin-type, 11S globulin, glycinin)	60, 37 (fragment)	Yes
Ara h 5	Profilin	15	No
Ara h 6	Conglutin (2S albumin)	15	Yes
Ara h 7	Conglutin (2S albumin)	15	Yes
Ara h 8	Pathogenesis-related protein, PR-10, Bet v 1 family member	17	No
Ara h 9	Nonspecific lipid transfer protein type 1	9.8	Yes
Ara h 10	Oleosin	16	Yes
Ara h 11	Oleosin	14	Yes
Ara h 12	Defensins	8	
Ara h 13	Defensins	8	
Ara h 14	Oleosin	17.5	Yes
Ara h 15	Oleosin	17	Yes
Ara h 16	Nonspecific lipid transfer protein 2	8.5	Yes
Ara h 17	Nonspecific lipid transfer protein 1	11	Yes

12.2.1 Primary Major Allergens: Storage Proteins

Ara h 1 is a 7S-globulin of vicilin-type and Ara h 3 a 11S-globulin, both members of the cupin-super family. Ara h 2, Ara h 6, and Ara h 7 are 2S-albumins and belong to the prolamin-super family (Radauer et al. 2012). As opposed to Ara h 7, Ara h 2 and Ara h 6 possess significant sequence homology. Though they belong to different protein families, Ara h 1, 2, and 3 exhibit high serological cross-reactivity and thus complicate the diagnostics of individual storage proteins (Bublin et al. 2013).

The storage proteins are the major allergens in primary peanut allergy. Sensitization to storage proteins are mainly found among patients who have suffered from a childhood peanut allergy. In a large, multicenter study including both children and adults (Ballmer-Weber et al. 2015), IgE specific to storage proteins was found exclusively in patients whose allergy had developed before the age of 14. Specific IgE against Ara h 2 and Ara h 6 was present in 76–96 % of children suffering from peanut allergy in the USA, Central and Northern Europe, but only in 42 % in Spain. For Ara h 1, the rate falls between 63 and 80 %. For Ara h 3 the rate is lower, and for Ara h 7 it merely adds up to 43 % (Codreanu et al. 2011; Vereda et al. 2011), defining it as a minor allergen.

12.2.2 Primary Minor Allergens: Oleosins

Oleosins are considered structure proteins of plant cells and function as potential allergens in legumes, seeds, and tree nuts. Their three-part form, similar to that of a hair

needle, with ambiphilic (both hydro- and lipophilic) ends and an extended hydrophobic domain, situated in the oil-matrix, contributes to the formation and stability of oil particles (oleosomes) and prevents the clotting of individual lipid drops. Several purified, native, or recombinant oleosin-isoforms of the peanut 14 (Ara h 11), 16 (Ara h 10), and 18 kDa are available. It has been shown that they can interact with one another in order to create oligomers, which are larger complexes (Cabanos et al. 2011).

Sensitization to oleosins probably only affects a minority of peanut allergy sufferers, but exact statistics are not known. As watery extract fluids of the nut contain little to no oleosins, this diagnostic gap complicates the identification of the affected patients (Aalberse et al. 2013).

Both storage proteins and oleosins are highly resistant to heat and digestion and thus relevant as primary food allergens (◉ Fig. 12.2).

12.2.3 Secondary Allergens: nsLTPs and Cross-Reactive Aeroallergens

Ara h 9, a nonspecific lipid transfer protein (nsLTP), is known as a secondary food allergen, especially in Mediterranean countries. The (secondary) sensitization/cross-reaction is most likely caused by other nsLTPs. Probably Pru p 3 in the peach fruit initiates the sensitization through skin contact. nsLTPs are also heat and digestion resistant; therefore, the affected patients may develop systemic symptoms (Petersen and Scheurer 2011).

Sensitizations to the Bet v t-homolog PR-10-protein Ara h 8, the profilin Ara h 5 and glycoproteins (CCD) are usually interlinked with cross-reactivity to pollen allergens. The sensitization rates vary depending on the regional pollen exposure. The birch tree predominance induces a distinct north–south pattern for cross-reactivity to Ara h 8; in regions with stronger grass, pollen exposure increased cross-reactive IgE against Ara h 5, and CCD-containing peanut extracts can be expected. The corresponding proteins are sensitive to heat and digestion, therefore since raw peanuts are generally not consumed, the pollen-associated peanut allergies only rarely account for symptoms, which are predominantly local oropharyngeal in nature.

12.3 Clinical Data Concerning Molecular *Diagnostics*

Peanut is the most commonly researched food allergen in clinical studies concerning the relevance of molecular allergy diagnostics. To date, studies attempted a better clinical interpretation of the specific serum-IgE-concentration against single allergens, specifically:

- A stronger association between specific IgE-sensitization profiles and clinical allergic reactions (risk rate for clinical/systemic reactions, odds ratio, OR)
- An increased diagnostic sensitivity and/or specificity (as shown in receiver operating characteristics-curves, ROC-curves)

- More accurate predictions ("predictive value") and calculable cutoff values/decision points for a positive (facilitated by the "positive prediction value", PPV) or negative prediction (facilitated by the "negative prediction value", NPV) of clinical reactions

In an earlier study, sensitization to one of the storage proteins Ara h 1–3 among children led highly significantly more often to systemic and severe clinical symptoms compared to sensitization to only Ara h 8 but to none of the storage proteins (Asarnoj et al. 2010). In a subsequent study encompassing 144 children and adolescents, it was found that a sensitization to only Ara h 8 without IgE against Ara h 1–3 always indicates tolerance to peanut. In only one child with systemic symptoms during the provocation, sensitization to Ara h 6 without IgE against Ara h 1–3 could be identified in the post hoc analysis of the sensitization spectrum (Asarnoj et al. 2012a). Several case reports in literature show patients with systemic reactions after contact with peanut and a sensitization to Ara h 6 without detectable IgE against Ara h 1, and 3 (Asarnoj et al. 2012b). In one rare observation, a 16-year-old female patient, who was mono-sensitized to Ara h 8, developed an anaphylactic reaction after consuming a large amount of peanuts (Glaumann et al. 2013).

An Australian study evaluated the benefits of measuring specific IgE against Ara h 2 among infants with a positive prick test against peanut to predict a clinical allergy. A model calculation in which only children with Ara h 2-specific IgE between 0.1 and 1 kU_A/l were admitted for the provocation test and children with Ara h 2-specific IgE >1 kU_A/l were considered allergic, the necessity for an additional provocation test for 95 children could be minimized to 44 children and therefore be reduced by half. The rate of false-negative results, regarding the Ara h 2-specific IgE diagnostics, amounted to 5 %, the rate of false-positive results to 3 % (Dang et al. 2012). Nineteen out of 100 children, who are identified as allergic, had IgE levels against Ara h 2 lower than 0.35 kU_A/l. Five of these did not have detectable antibodies against Ara h 1 or 3, none against Ara h 8 or 9.

Numerous different studies analyzed the diagnostic sensitivity and specificity of various IgE levels against Ara h 2 for the prediction of an allergic reaction. Eller and Bindslev-Jensen (2013) calculated a diagnostic specificity of 100 % and a sensitivity of 70 % for a cutoff value of 1.63 kU_A/l among 205 Danish patients aged 1–26; Nicolaou et al. (2011) determined a sensitivity of 93 % and a specificity of 100 % for a cutoff of 0.55 kU_A/l among 81 British children. In a French study, only 7 out of 166 peanut-allergic children and adolescent were not sensitized to Ara h 2. For a cutoff value of 0.23 kU_A/l, a diagnostic sensitivity of 93 % and a specificity of 96 % were calculated. The analysis of Ara h 6-specific IgE provided added value (Codreanu et al. 2011).

One of the largest cohort studies (210 children suspected to be peanut allergic) took place in Germany and examined patients using standardized peanut provocations (Beyer et al. 2015). During this study, probability curves (◉ Fig. 12.3) for a

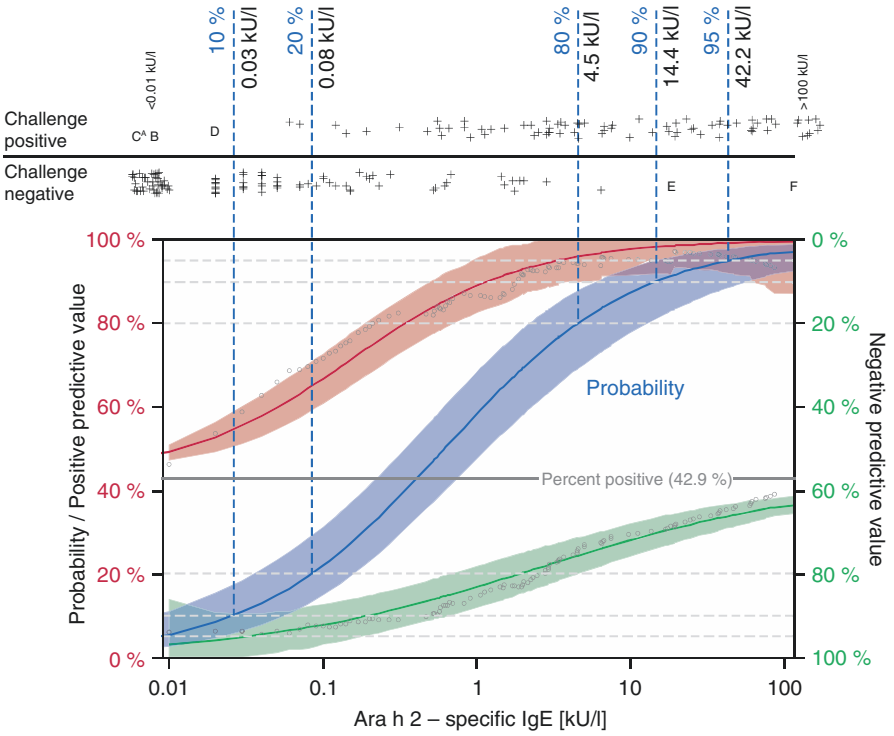

Fig. 12.3 Results concerning probability for a positive peanut food challenge by Ara h 2-specific IgE. Sigmoidal calculated probability for a positive peanut challenge resulting from Ara h 2-specific IgE concentration (*blue line* and band). Estimated IgE levels at given probabilities (5, 10, 20 % and 80, 90, 95 %) above figure (*dashed lines*). Estimated positive (*left axis, red line,* and band) and negative (*right axis, green line*, and band) predictive value, and real cases (*gray dots*). Actual IgE levels by challenge outcome above figure (*plus sign*). *Letters* indicate positive challenge outcomes (*A–D*) with Ara h 2-specific IgE <0.03 kU_A/l (below the 10 % probability) and negative challenges (*E, F*) with Ara h 2-specific IgE >14.4 kU_A/l (levels above 90 % probability (Adapted from Beyer et al. 2015)

clinically relevant allergy to Ara h 2 were first calculated. The cutoff value for a 95 % prediction of peanut allergy using Ara h 2-specific IgE amounted to 42 kU_A/l (ImmunoCAP Singleplex, ThermoFisher). This high value resulted from unselected inclusion of children, leading to the participation of patients with higher Ara h 2-specific IgE levels. Two of these children were tolerant to peanut during the provocation despite their high Ara h 2-specific IgE levels (18 kU_A/l and >100 kU_A/l, respectively). In contrast, 4 patients without sensitizations to Ara h 1–3 showed clinical symptoms, presumably due to clinically relevant sensitizations to other single allergens not identified. Specific IgE to Ara h 6 was not tested.

Take-Home Message of the Multicenter Peanut Study (Beyer et al. 2015)

- Ara h 2-specific IgE currently shows the best association with systemic reactions to peanut in the context of oral provocation.
- In order to predict a positive provocation with a 95 % probability, the Ara h 2-specific IgE must have a value >42.2 kU_A/l – an uncommon constellation and thus only useful in similarly extreme cases.
- In order to predict a negative provocation a 90 % probability, the Ara h 2-specific IgE must have a value <0.03 kU_A/l – apart from deviating individual cases.
- Due to exceptions, a definite 100 % prediction via Ara h 2-specific IgE is not possible. Therefore, the clinical relevance of allergen-specific IgE levels (e.g., against single allergens of legumes) must be determined by the attending physician.

It was previously reported that young children (below 24 months of age) with a sensitization to peanut recognize predominantly seed-storage proteins particularly Ara h 1 (Trendelenburg et al. 2014). Identified with the slightly less sensitive Microarray-System ISAC (ThermoFisher), these IgE sensitizations were partly also clinically relevant, though specific IgE against Ara h 2 was not determined. In addition, the benefits of analyzing IgE levels against the three storage proteins (Ara h 1–3) in adult patients, who probably developed their allergy in childhood years, could be shown among 74 Swedish patients (Movérare et al. 2011).

On the other hand, a subproject of the EuroPREVALL study throughout Europe identified adults, whose peanut allergy had only manifested itself from an age of 14 or older, who did not show sensitizations against Ara h 1–3 or Ara h 6 (Ballmer-Weber et al. 2015). The majority of these adults had strikingly low titers of specific IgE to the total extract of the peanut. These patients were often sensitized against the nsLTP Ara h 9 in Southern Europe. Several patients did not show specific IgE to any of the tested components. The reason for this could be sensitization against oleosins (Ara h 10 and 11); however, their potential relevance can only be assumed, as they have until now not been available for IgE diagnostics.

These data show that in general, patients from Middle Europe, who developed their peanut allergy up to adolescence, probably do not have a clinically relevant allergy, if they lack IgE against storage proteins Ara h 1–3 and Ara h 6.

Due to varying prediction values and the fact that some relevant peanut allergens are still unavailable for diagnostic purposes, the determination of the anaphylaxis risk is not possible solely through the determination of IgE against Ara h 2.

Confounding factors such as age, underlying medical conditions, total IgE, or sensitization to other allergens inevitably are disregarded during cohort analyses, which may lead to enormous deviations and in turn, create false-positive results.

This was once again demonstrated in a Berlin study, during which all children with suspected peanut allergy were challenged with peanut, regardless of their level of peanut-specific IgE. Twenty-seven percent of the children with detectable levels

of specific IgE against Ara h 2 were tolerant and partially showed high levels of Ara h 2-specific IgE (Lopes de Oliveira et al. 2013).

In Southern Europe, specific IgE against the lipid transfer protein Ara h 9 is also considered to possess a predictive value for a systemic allergic reaction (Krause et al. 2009). The majority of patients in these regions are not sensitized against Ara h 2, but against Ara h 9 (Vereda et al. 2011).

12.4 Diagnostics with Peanut Allergens

12.4.1 Available Single Allergens

Specific IgE antibodies can be determined against the crude peanut extract, the storage proteins Ara h 1, h 2, h 3, and h 6, against the nsLTP Ara h 9, and against the PR-10-protein Ara h 8 (◉ Fig. 12.2).

12.4.2 Potential Benefits of Molecular Diagnostics with Peanut Allergens

When IgE sensitization is identified through the determination of single peanut allergens, the test properties are altered (without necessarily impacting on the clinical relevance of the test results) (Matricardi et al. 2016). Furthermore, it allows the detection of marker allergens and may provide indications of primary sensitization:

- The assay sensitivity is improved through the introduction of underrepresented or absent peanut allergens (lower "limit of quantitation", LoQ).
  Examples: Ara h 8, Ara h 10/11 (the latter ones not yet available for diagnostics).
- The analytical specificity (selectivity) of the determination of IgE is augmented through the determination of single allergens in comparison to whole extract diagnostics. This is especially appropriate for risk associated peanut allergens, which are rather interlinked with clinical reactions (Ara h 2), as well as for low risk peanut proteins, which are connected to serological, yet clinically irrelevant cross-reactions (Ara h 8).
- Markers for general cross-reactions connected with peanut allergens include in particular Ara h 8 (Bet v 1-associated cross-reactivity), Ara h 5 (profilin-associated cross-reactivity), MUXF3 (CCD-induced cross-reactivity). They are responsible for the unsatisfactory specificity of peanut extracts regarding the detection of differentiated IgE sensitization.
- Peanut allergens (Ara h 1, 2, or 3) do serve as an indicator for a primary, species-specific sensitization, which developed in childhood years, so long as the specific IgE against corresponding storage proteins (2S-albumins, 7S- and 11S-globulins) of other legumes (e.g., soy) or other nuts (tree nuts, drupes, and capsule fruits) or seeds is considerably lower. A number of storage proteins for specific IgE

diagnostics are still missing, which would be necessary in order to systematically differentiate dominant, primary sensitizations from serological cross-reactions.

12.4.3 Procedure for Diagnosing Peanut Allergy in Childhood (<14 Years)

Various diagnostic questions arise depending on medical history and preliminary findings:

A. Desire for exclusion of peanut allergy (e.g., among patients with atopic dermatitis or other food allergies), prior to the consumption of peanut-containing products (◉ Fig. 12.4)
B. Incidental finding of a sensitization (e.g., raised IgE against peanut in the panel- or screening test) (◉ Fig. 12.5)
C. Allergic reaction following peanut contact or consumption (◉ Fig. 12.6)

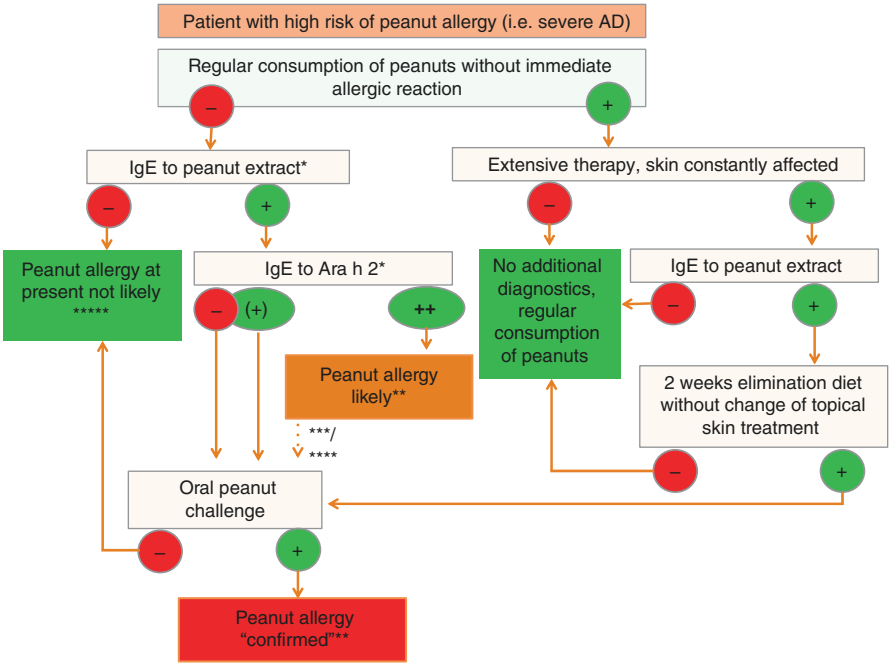

Fig. 12.4 Model of a diagnostic algorithm for excluding the possibility of peanut allergy when suspected. * Consider tests in parallel, ** prescribe emergency kit/drugs, *** consider oral challenge test to confirm the diagnosis, **** oral challenge test at appropriate intervals to detect tolerance development, ***** in case of sensitization without clinical symptoms regular consumption of peanut products 3×/week recommended

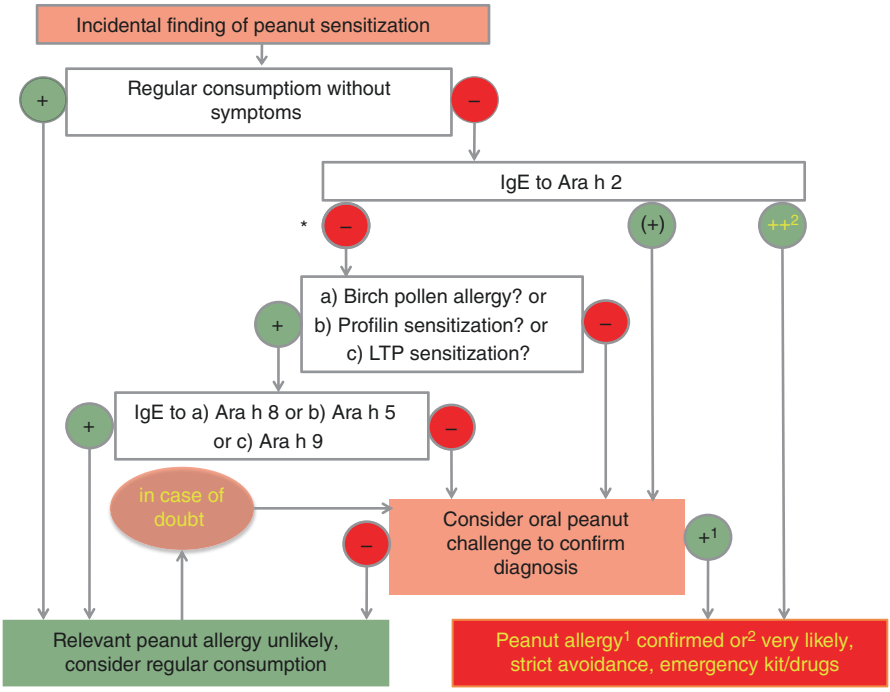

Fig. 12.5 Model of a diagnostic algorithm for sensitizations detected by chance (* For maximum diagnostic reliability IgE against Ara h 1, 3, 6 should be considered)

Ad A

IgE against whole peanut extract is well suited as a screening parameter (especially for exclusion) of peanut allergy: undetectable peanut-IgE has a high negative predictive value (rare exception: relevant sensitizations against oleosins Ara h 10/11). A positive IgE result is only clinically relevant if the symptoms correspond (low diagnostic specificity). In the case of negative-specific IgE, an additional prick test (e.g., prick-to-prick test with native peanut) serves as a sensitization verification or exclusion criteria. If positive, an oral provocation should be considered.

Ad B

In clinical practice, positive IgE results against peanut may be recorded accidentally. A stepwise approach (◉ Fig. 12.4) takes into account potential consequences and the cost-benefit ratio of diagnostics. The most important initial question is concerned with the regular (e.g., more than once a month) and recent (e.g., within the period of the last 6 weeks) consumption of a relevant quantity of peanut.

Ad C

The determination of IgE level against Ara h 2 is an important parameter in patients suspected to have a primary peanut allergy, which developed in

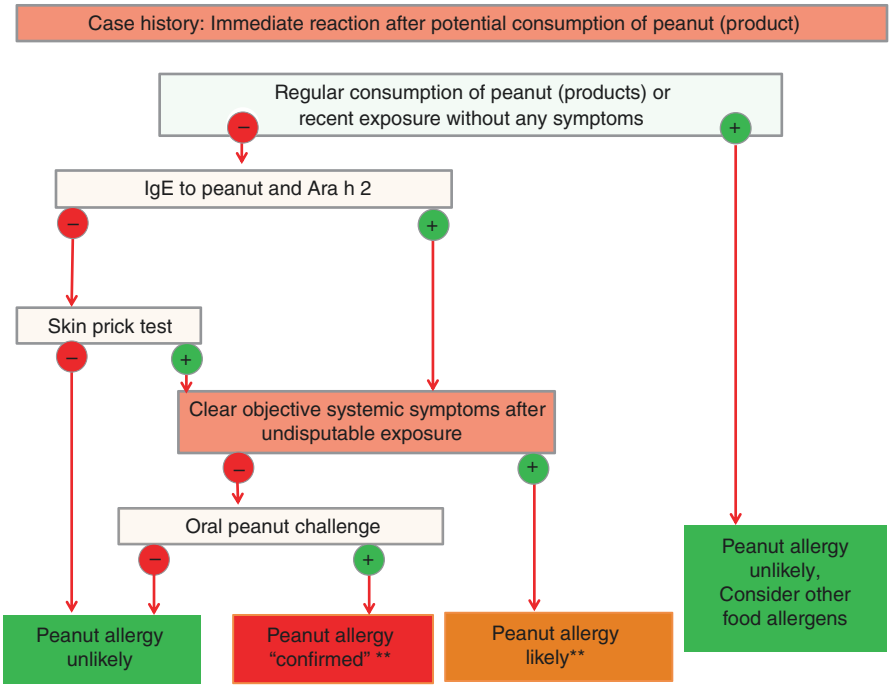

Fig. 12.6 Diagnostic algorithm for immediate type reactions following peanut consumption "** prescribe emergency kit/drugs"

childhood years. A clinically relevant allergy is probable in the case of significantly raised specific IgE and positive patient history of immediate allergic reactions following peanut consumption. However, the published data are heterogeneous and the calculated cutoffs (between 1 and 42 kU_A/l for Ara h 2-specific IgE) resulted in different diagnostic sensitivities and specificities in the examined patient populations. Nevertheless, probability curves were now calculated for a clinically relevant allergy against Ara h 2. The specific IgE against Ara h 6 may be a similarly relevant parameter; however, there is insufficient data available in comparison to Ara h 2.

12.4.4 Common Peanut Cross-Reactions Regarding Birch Pollen Sensitization

If birch pollen–associated sensitization is suspected, determining the IgE levels against Ara h 8 and Ara h 2 is useful. If Ara h2 is negative and Ara h 8 positive, this indicates a Bet v 1-related cross-reactivity with low clinical relevance. Cross-reactions induced by CCD or profilins may present further reasons for positive IgE results.

12.4.5 Less Common Sensitization Patterns in Peanut Allergy

Evidence of sensitization against Ara h 1 and 3 is often not necessary, as a high cross-reactivity exists between the storage proteins (Bublin et al. 2013) and monosensitization against Ara h 1 and/or 3 is rare. In cases where undetectable or low IgE against Ara h 2 raise doubt, a double-blind oral provocation with peanut can provide clarity to the diagnosis. If IgE is undetectable to all of the storage proteins, the possibility of a clinically relevant peanut allergy is relatively unlikely, yet cannot be excluded if clinical symptoms suggest otherwise. A diagnostic gap is present among infants (Trendelenburg 2014) and adults who developed their allergy after adolescence and in regard to oleosins Ara 10/11. Furthermore, IgE levels of patients from Mediterranean regions should additionally be tested against the nsLTP Ara h 9.

12.5 Cross-Reactive Allergens

Clinically relevant cross-reactions are predominantly induced through storage proteins. Reactions are possible to legumes, such as lupines and lentils, but also to nuts, such as hazelnuts, walnuts, or seeds, such as sesame. Serological cross-reactions must be critically evaluated in order to prove clinical relevance. For instance, the detection of IgE antibodies to soybean is mostly irrelevant for peanut allergy sufferers.

12.6 Conclusions: Relevance in Daily Clinical Practice

Molecular allergy diagnostics (Matricardi et al. 2016) has considerable significance in the diagnostic procedure of peanut allergy:

- Numerous sensitizations against peanut extracts in our latitudes evolve from pollen-associated cross-reactions, which can be differentiated with IgE measurement against available marker allergens (e.g., Bet v 1-homologs Ara h 8, CCD MUXF3, Profilin Phl p 12).
- The corresponding clinical reactions are often mild and mostly limited to local reactions of mouth- and throat regions.
- For peanut allergy sufferers from the Mediterranean regions, Ara h 9 is included in IgE diagnostics as nsLTP can be associated with systemic reactions.
- Considerably raised specific IgE against stable storage proteins like Ara h 2 (and probably Ara h 6) are often associated with systemic reactions and a clinically relevant peanut allergy.
- In patients with reliable systemic reactions to peanut and sensitization especially to Ara h 2, a further oral food allergen provocation is not necessary.
- Storage proteins are most likely not the responsible major allergens, if the peanut allergy only develops in adult years.

- If uncertain, the clinical diagnosis of peanut allergy can be verified by an oral provocation due to the following reasons:
  - Some patients with Ara h 2-specific IgE may be tolerant and some affected individuals may react systemically despite lacking Ara h 2-specific IgE for peanut.
  - Not all relevant peanut allergens are available for diagnostics.
  - Traceable specific IgE concentrations correspond to a sensitization (allergic disposition), which is only clinically relevant in connection with the corresponding symptoms.

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