

ORIGINAL ARTICLE

Assessment of the tolerance to lupine-enriched pasta in peanut-allergic children

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Summary

Background Reports of allergy to lupine derivatives (as *de novo* sensitization or cross-reactivity in subjects allergic to peanut) are increasing as their use in food products increases. **Objectives** The aim of this study was to assess: (1) lupine tolerance in a group of children allergic to peanut, using lupine enriched-pasta instead of raw flour as has been done in previous clinical studies; (2) whether technological treatments of lupine modify its cross-reactivity or co-sensitization with peanut; (3) the role of lupine seed proteins in sensitization, and (4) to identify the eliciting doses (EDs) by using double-blind, placebo-controlled food challenges (DBPCFC).

Methods Twelve patients with a history of clinical allergic reactions to peanut were evaluated by skin prick tests (SPTs), the ImmunoCAP® test, immunoblotting, and DBPCFC. The 12 selected subjects were included in a trial where lupine-enriched pasta and placebo pasta were administered in a DBPCFC protocol.

Results Positive clinical reactions were observed in two children, the EDs being 0.2 and 6.4 g of pasta, corresponding to 50 mg and 1.6 g of lupine proteins, respectively. β -conglutinin was the protein most involved in SPT positivity.

Conclusion Lupine-enriched pasta can be tolerated by most subjects suffering from peanut allergy, but a sizeable minority (2/12 of them in this case) can develop potentially dangerous clinical reactions. Information about possible reactions to lupine derivatives by those allergic to peanuts must be included in the labelling of lupine-enriched products to protect consumers at risk.

Keywords cross-reactivity, food allergy, lupine allergy, peanut allergy, technological treatments

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Introduction

Lupine flour is increasingly used in the food industry because of its protein-rich composition and good technological characteristics. Lupine (*Lupinus* spp.) is a member of the *Fabaceae* (ex *Leguminosae*) family which includes the well-known allergen peanut (*Arachis hypogaea*). Used to add protein and fibre and to improve texture, lupine is also a useful alternative to soybean when non-genetically modified food ingredients are sought (e.g. in baby foods).

The first reported case of allergy to lupine involved a girl with known peanut allergy who experienced urticaria and angio-oedema after ingesting a pasta fortified with sweet lupine seed flour [1]. Since then, lupine allergy has

mostly been reported in patients with allergies to other legumes, particularly peanut [1–4], but some cases of primary lupine allergy have also been described [5–10]. The few studies assessing the risk of cross-reaction between peanut and lupine showed that the risk is quite high, unlike that between peanut and other legumes. In double-blind challenge studies, 30–68% of patients allergic to peanuts showed positive reactions to lupine flour [4, 11]. Cross-reactions are a clinically significant problem in children too [12]. On the basis of findings by the European Food Safety Agency [13], the European Union included lupine among allergenic foods for which labelling is mandatory [14].

In Italy, lupine flour and lupine protein concentrate are being increasingly used in bakery and pasta formulations,

in particular in gluten-free products, where lupine derivatives produce better elasticity, texture, and flavour than soybean substitutes [15]. Doxastakis et al. [16] showed that spaghetti fortified with 5% of lupine protein isolate had a colour and rheological features comparable with pasta produced with gluten, and also had good cooking qualities.

Although the technological quality of lupine flour is unquestionable, its possible allergenic activity in subjects allergic to peanut or other legumes must also be considered. Most authors considering in clinical trials the cross-reactivity between peanut and lupine used raw lupine flour even for oral challenges. This cannot be considered a suitable approach for two reasons: (1) raw flour from legume should not be used in oral challenges as well as in human nutrition for the presence of anti-nutritional factors [17]; (2) technological treatments and/or cooking might modify the allergenicity of lupine protein, either increasing or decreasing it.

For these reasons, we have evaluated the tolerance of a group of peanut-allergic children to lupine-enriched pasta, compared with a pasta having similar characteristics but produced without lupine derivatives (pasta placebo).

Materials and methods

Legumes

Included in fresh food skin prick test (SPT) and immunoblotting tests were commercial roasted peanut (*A. hypogaea*), lupine (*Lupinus albus*) protein concentrate, and two kinds of pasta, finely ground and prepared as described in the section regarding the specific test.

Purified lupine proteins

α -, β - and γ -conglutins were purified from the total lupine protein extract using a combination of gel filtration chromatography to remove undesired components and ion-exchange chromatography to fractionate the three protein families, as detailed by Magni et al. [18].

δ -conglutin was purified using several selective precipitations as described by Sironi et al. [19]. Quality of purified proteins was verified by SDS-PAGE.

Experimental and control pasta for oral challenge

Pasta used in oral challenge tests was prepared as small macaroni. Lupine-enriched pasta (experimental pasta) contained lupine protein isolate 37%, semolina 36%, lupine flour 15%, wheat flour 12%, with a final lupine protein concentration of 25%. Placebo pasta contained semolina 50%, wheat flour 42%, and carrot 8%.

Patients

The design and aims of the study were authorized by the institutional review board. Patients and their parents were provided with full information before they signed the consent form.

Patients admitted for immediate symptomatic onset after peanut exposure between 1 December 2006 and 30 June 2007 were considered when they presented for follow-up visits. According to hospital internal protocols, children were diagnosed as allergic to peanut if:

- They tested sensitive to peanut proteins (SPT and/or RAST positive response) *and* were positive to oral challenge.
- Or, when oral challenge was contraindicated, they met the first criterion *and* had a history of anaphylaxis after ingestion of peanut [20].

Previous clinical reactions to carrots and/or wheat proteins were considered criteria for exclusion.

Skin prick test and fresh food skin prick test

SPTs were performed using both commercial (SPT) and freshly prepared allergens (FFSPT); as it was part of the placebo, carrot was included in the tests.

Commercial extracts included peanut (*A. hypogaea*, Lofarma, Milan, Italy), and carrot (*Daucus carota*, ALK-Abellø, Copenhagen, Denmark). Commercial lupine reagent was not available. Lupine protein concentrate, uncooked lupine-enriched pasta and placebo-pasta were ground and suspended in the SPT vehicle to obtain a 5% final protein concentration. The protein concentration of all samples was experimentally determined by the Kjeldahl method [21–22].

The vehicle contained 2% mannitol, 5.9% NaCl, 58% glycerol, and 0.4% phenol; it was sterilized by microfiltration.

Weal diameters were measured by a caliper disk marked in millimetres 15 min after testing, and a test was considered positive when the weal was wider than that of a negative control by at least 3 mm [23]. Histamine phosphate (Lofarma), 10 mg/mL in 50% glycosaline solution and SPT vehicle alone were used as positive and negative controls, respectively.

Specific immunoglobulin E determination by ImmunoCAP[®]

Specific IgEs were measured in patients' sera from venous blood samples obtained at the time of enrolment. Levels of total IgE levels and of IgEs specific for peanut and known lupine allergens were measured by an ImmunoCAP[®] test (Phadia, Upssala, Sweden). Allergen-specific IgE titres above 0.35 kU/L were considered positive.

Double-blind, placebo-controlled food challenges

Peanut allergy was confirmed with blinded challenges. Challenge meal volume based on the highest protein amount set for office procedure yielded up to 36 g of dry toasted peanut pounded in a mortar in doses refracted in 2.4, 4.8, 9.6, and 19.2 g/days. Strained apple was added as a mask and used as placebo. Placebo and allergen challenges were administered on same days over 4 h at 30 min intervals.

Similar challenges with lupine-enriched pasta were administered to all children, at the second scheduled visit, 15–30 days after SPT. Placebo or experimental pasta were fed to the patients on separate days at 30-min intervals over 4 h, doubling the dose of lupine-fortified or control pasta at each feeding. Pasta was cooked in boiling water (100 g in 1 L of 1% NaCl) for 9 min and made palatable by adding extra-virgin olive oil (3–10 g according to the dose) immediately before the oral test. The doses administered were 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 g of macaroni (the last dose at 3.5 h), totaling 12.7 g of lupine protein for children under 13, with a ninth dose of 51.2 g of pasta at 4 h for children over 13 years of age for a total of 25.6 g of lupine protein. The same amount of carrot-containing pasta was administered on another day as a placebo. Challenge was discontinued at the first onset of symptoms accompanied by objective signs or, when there was no response, at the eighth or ninth dose.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting

The pattern of specific IgE binding to legume proteins was determined by immunoblotting using patients' sera. Specificity of IgE-binding was verified by using 10 control sera (healthy subjects).

Legume flours were suspended in sample buffer (containing 0.125 M Tris-HCl pH 6.8, 3.75% glycerol, 1% SDS, with or without 5% β -mercaptoethanol) at a final protein concentration of 1.5 mg/mL.

Legume proteins were then separated in 15% polyacrylamide gel according to Restani et al. [24], and transferred from the gel onto a polyvinylidene difluoride membrane (PVDF, Immobilon P, Millipore, Bedford, MA, USA) by electrophoretic elution. The transfer buffer was Tris 25 mM, glycine 193 mM and methanol 20%. PVDF membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (Bio Rad, Milan, Italy) in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.05% Triton-X. The membranes were washed with 10 mL of 0.25% gelatin (BioRad) solution containing 0.3 mL of allergic patients' serum. Antigen-IgE complexes were detected using goat anti-human IgE antibodies labeled with alkaline phosphatase (Sigma Aldrich, Milan, Italy). After incubation in bromochloroindolyl-phosphate/nitroblue tetrazolium solution, an intense precipitate developed at the site of the enzyme binding. The immunoreactive bands were quantified by means of a gel scanner (Sharp JX-330, Pharmacia Biotech, Milan, Italy) and the Image MasterTM 1D Software, which measures the average density of pixels over the band length and integrates it over the band width.

Classes of positive reactions were defined on the basis of an arbitrary scale of densitometric values, and six classes of reactivity were identified; from our previous experience, classes ≤ 2 were considered non-specific.

Results

One girl and 11 boys were recruited for this study (Table 1). Their age ranged between 2.10 and 17.75 years, mean $\pm$ SD 10.74 $\pm$ 4.45 years; median 10.54 years.

Table 1. Clinical characteristics of children included in the study

Child	Sex	Age (months)	Symptoms after peanut ingestion at referral	Cumulative dose eliciting symptoms at challenge	Total IgE (mIU/L)	DBPCFC with lupine pasta
1	Female	104	Urticaria, rhinitis	16.8	94.2	No reaction
2	Male	145	Oral urticaria and angio-oedema	7.2	523	Oral urticaria
3	Male	213	Urticaria, angio-oedema	2.4	721	No reaction
4	Male	156	Anaphylactic shock	NP	1231	No reaction
5	Male	101	Anaphylactic shock	NP	321	No reaction
6	Male	74	Urticaria, angio-oedema	7.2	1895	Urticaria
7	Male	135	Gastrointestinal symptoms (vomit)	7.2	163	No reaction
8	Male	169	Urticaria, angio-oedema	16.8	298	No reaction
9	Male	102	Angio-oedema, asthma	7.2	254	No reaction
10	Male	204	Urticaria	16.8	1052	No reaction
11	Male	25	Urticaria, rhinitis	16.8	NA	No reaction
12	Male	118	Urticaria, asthma	16.8	61	No reaction

NA, not available: insufficient serum for determination; NP, not performed: history of anaphylaxis; DBPCFC, double-blind, placebo-controlled food challenges.

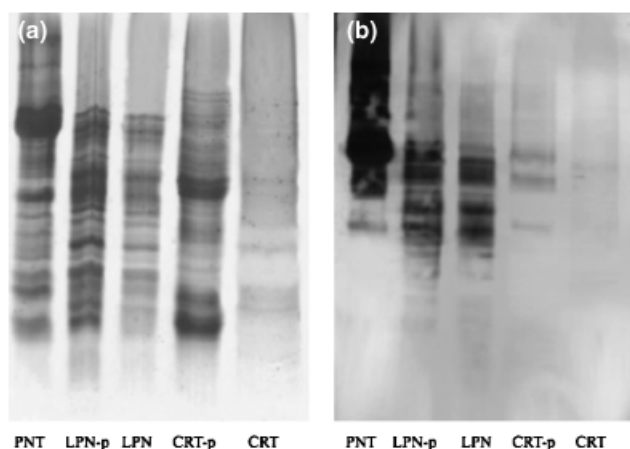


Fig. 1. SDS-PAGE (a) and immunoblotting (b) of samples included in this study. The immunoblotting shown in the figure was obtained by incubating a membrane containing the same protein pattern of Fig. 1a with the serum of Child 2. PNT, peanut; LPN, lupine; LPN-p, lupine-enriched pasta; CRT, carrot; CRT-p, carrot-enriched pasta.

Allergic symptoms reported after peanut ingestion were anaphylactic shock ($n = 2$), asthma ($n = 2$), angio-oedema ($n = 5$), urticaria ($n = 8$), allergic rhinitis ($n = 2$) and gastrointestinal symptoms in a single patient. The subjects having a history of anaphylactic shock (Children 4 and 5) were not challenged again with peanut before their inclusion in the study. According to the EAACI guidelines [25], the child younger than 3 years (Child 11) was diagnosed by open challenge and the remaining nine by double-blinded challenge. All children with peanut allergy were atopic, as witnessed by high total IgE values.

Figure 1 shows the electrophoretic pattern of samples considered in this study and, as an example, the immunoblotting obtained with the serum from Child 2, who had a positive response to DBPCFC. The intensity of reaction obtained in each immunoblotting was classified according to the densitometric analysis. The electrophoretic analysis was performed using both reducing and non-reducing conditions (sample buffer with or without β -mercaptoethanol) but only non-reducing results are considered here, because they are the most appropriate to be correlated with other clinical tests, which do not use reducing conditions. Molecular aspects will be specifically discussed in another paper, in preparation.

The results of all tests performed are illustrated, child by child, in Fig. 2. The sensitivity to peanut was confirmed:

- in 12/12 children at SPT performed with commercial extract;
- in 11/12 children at FFSPT performed with fresh prepared reagent (negative result in Child 3);
- in 10/12 at ImmunoCAP[®] test (negative results in Children 1 and 12);
- in 11/12 in immunoblotting (Class ≤ 2 in Child 12);

Lupine-specific responses were determined:

- in 8/12 children at FFSPT performed with lupine protein concentrate (negative results in Children 1, 3, 4, and 7);
- in 7/12 children at ImmunoCAP[®] test (negative results in Children 1, 5, 7, 11, and 12);
- in 8/12 on immunoblotting (non-specific classes ≤ 2 for children 7, 10, 11, 12).

When lupine protein concentrate was present in the pasta, the pattern of positive response at FFSPT was similar, but further negative responses were found in Children 4 and 8.

The reactivity against purified lupine proteins, evaluated by FFSPT, was positive:

- in 5/12 children for α -conglutin (11S legumine);
- in 7/12 children for β -conglutin (7S vicilin);
- in 4/12 children for γ -conglutin (7S basic);
- in 3/12 children for δ -conglutin (2S albumin).

Only two children tested positive at DBPCFC with lupine-enriched pasta (Table 1), confirming the known fact that specific cutaneous and circulating sensitizations do not always translate into full-blown allergy. In particular challenge was positive:

- In Child 2, a 12-year-old boy with a history of severe persistent asthma and anaphylactic reaction after the ingestion of cashews, and urticaria-angio-oedema after peanuts; this child proved highly sensitive to peanut and lupine in all tests performed. Moreover, very large weals were obtained in FFSPT when purified α - and β -conglutins were tested. Symptoms included oral urticaria with throat pruritus on the first challenge dose (lupine protein content 0.2 g).
- In Child 6, a 6-year-old boy with former atopic dermatitis and immediate reactions to egg, kiwi fruit, nuts, and peanut, but no anaphylaxis to peanut; FFSPT was positive with β -conglutin. Symptoms included urticaria after the sixth dose (lupine protein content 6.4 g).

Discussion

The tolerance/intolerance to lupine flour and protein concentrates in subjects suffering from peanut allergy has been evaluated by various research groups; the most recent paper on this topic [26] showed that a small but significant number of children with peanut allergy are also allergic to lupine. The cross-reactivity between peanut and lupine proteins has been described in some other papers and all authors agree with the existence of possible risk of clinical reaction to lupine in subjects allergic to peanut [3–4, 8]. The critical point is how prevalent is this phenomenon, because percentages of

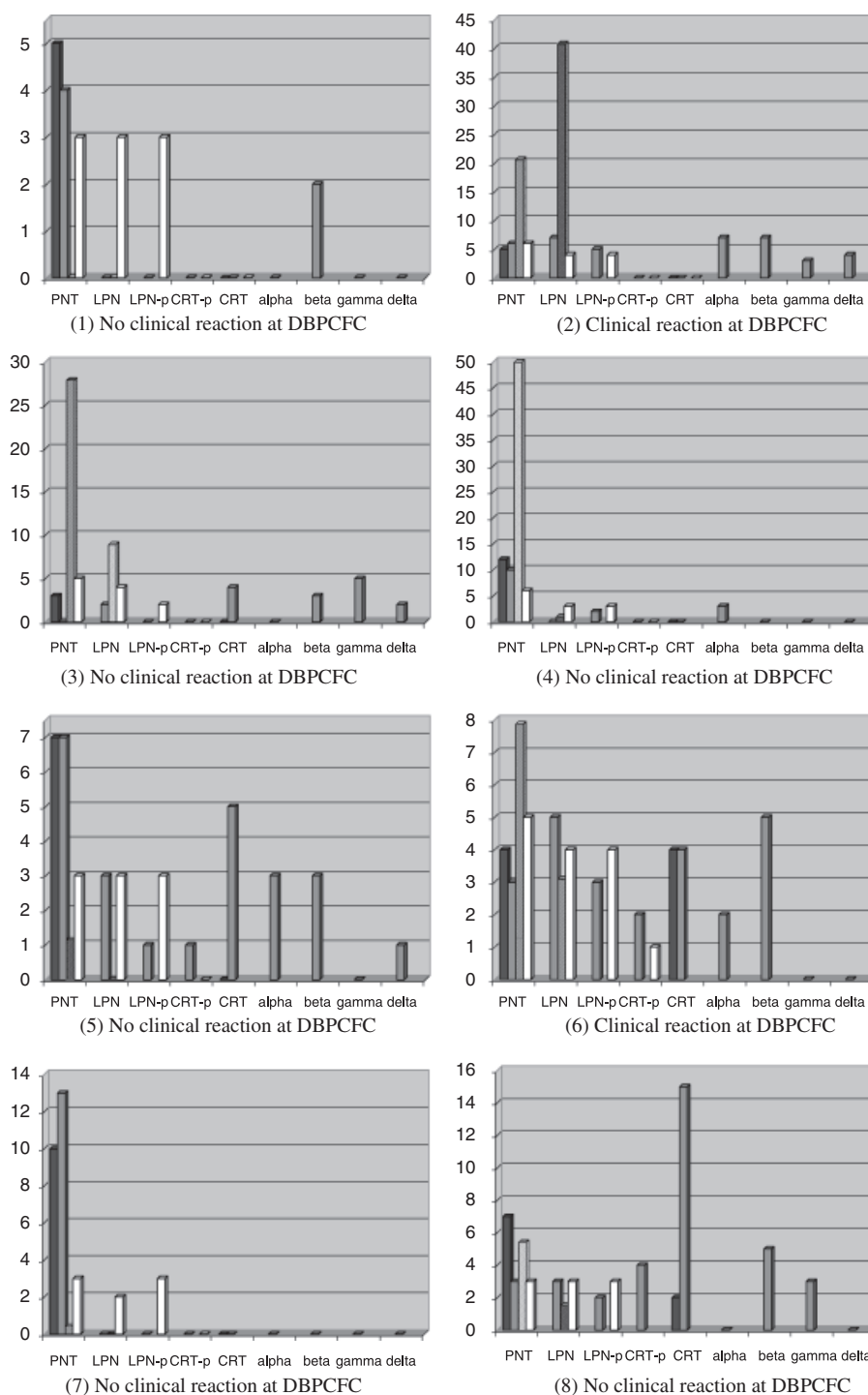


Fig. 2. Sensitization test pattern for the study sample. Every graph represents a single patient. Black bar: SPT (weal diameter in millimetres); grey bar: FFSPT (weal diameter in millimetres); hatched bar: ImmunoCAP® (kU/L); white bar: immunoblotting, expressed as class of reactivity. PNT, peanut; LPN, lupine; LPN-p, lupine-enriched pasta; CRT, carrot; CRT-p, carrot-enriched pasta; SPT, skin prick test. Ordinates are not to scale.

clinical cross-reactivity differ significantly according to the experimental conditions used.

The aim of this study was to evaluate the cross-reactivity between peanut and lupine proteins, using a product similar to that currently marketed, i.e. lupine-

enriched pasta. It is well known that technological treatments and/or cooking can modify the allergenic potential of raw ingredients, and in our opinion the use of raw flour should be avoided in oral challenge, even when as in the case of lupine the content of anti-nutritional compounds

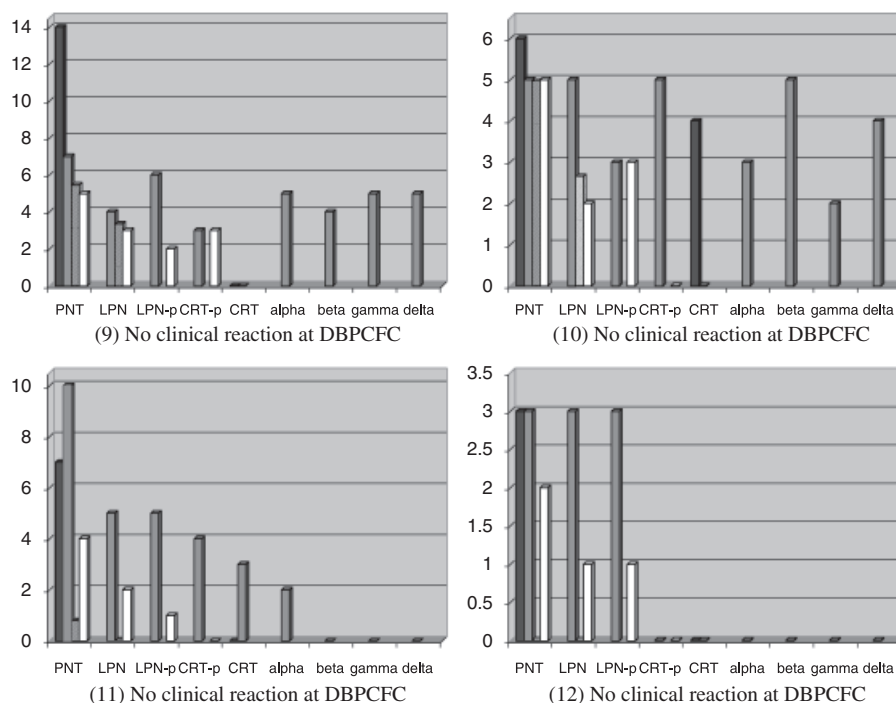


Fig. 2. Continued.

(lectines, anti-trypsin factors) is low. These factors are thermo-sensitive and their adverse effect is eliminated by cooking. The blind challenge was made possible through the use of a carrot-enriched pasta having comparable colour and organoleptic characteristics. As all children consumed carrot freely, the presence of sensitization to carrot at SPT in six children was considered clinically not significant.

Several considerations can be drawn from these data:

1. Legume sensitization pattern: there is a good agreement between responses in SPT and FFSPT, and between both these tests immunoblotting, but the level of circulating specific IgE determined by ImmunoCAP[®] is often in disagreement with other diagnostic tests (large weals with low IgE level, and vice versa). This indicates a low degree of agreement between legume ImmunoCAP determinations and other sensitization tests in this sample.
2. Whether there will be a reaction to lupine-enriched pasta cannot be predicted by the reported severity of symptoms after peanut ingestion.
3. In some cases, as in children 4 and 8, industrial food processing and/or cooking eliminated the clinical reactivity observed with fresh food in FFSPT, in contrast to the conclusions of Alvarez- Alvarez *et al.* [27], who reported that only autoclaving at 138 °C for 20 or 30 min was able to reduce or eliminate (respectively) the IgE binding to lupine proteins.
4. Our reactivity rate at oral challenge was similar to the 20% rate found by Shaw *et al.* [26] but lower than that found by Moneret-Vautrin (17% vs. 87.5%). This suggests that cooking or technological treatments may actually reduce clinical reactivity, but it is not able to abolish it, as has been shown previously [28].
5. The only children (2 and 6) who showed clinical reaction when tested at DBPCFC with lupine-enriched pasta have some similar characteristics: positive results in all tests against lupine proteins and lupine-enriched pasta, and weal diameters in FFSPT reactions against β -conglutin of 5–7 mm.
6. β -conglutin seemed the most important lupine allergen, confirming the inclusion of the homologous *Lupinus angustifolius* protein in the list of allergens from plant origin as *Lup an 1*. At present, no other lupine protein has been included in the list [29].
7. When all immunologic tests were positive vs. lupine total proteins (children 2, 3, 6, 8, 11, and 10), a specific reaction to β conglutin could be a marker of an increased risk of clinical reaction in children allergic to peanut. On the other hand, SPT reaction to this protein, in the absence of other positive tests, cannot be considered significant enough for a clinical reactivity (see children 8 and 10). Considering the pattern of sensitivity to peanut allergens of each child included in this study, it would be interesting to assess whether the sensitivity to Ara h 1, which is homologous to β -conglutin [30], could be a marker of

clinical cross-reactivity between the two legumes. This study is in progress.

In conclusion:

- concordance between different immunological tests is a necessary, but not sufficient, indication of clinically significant sensitivity;
- although lupine-enriched pasta was tolerated by 10/12 of the children allergic to peanut included in this study, if lupine flour is to be used increasingly in bakery and pasta production, the possibility of cross-reactivity between peanut and lupine should be reported in the labelling to avoid clinical reactions in consumers with peanut allergy.

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