

Original article

Allergenicity of different apple cultivars assessed by means of skin prick test and sensitisation to recombinant allergens Mal d 1 and Mal d 3 in a group of Italian apple-allergic patients

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Summary To evaluate differences in skin prick test response to apple cultivars in patients with apple allergy, 19 patients (10 adults and 9 children) underwent prick-to-prick skin prick test with eleven commercial and noncommercial apple cultivars, and evaluation of specific IgE to apple and recombinant apple allergens Mal d 1 and Mal d 3. The results show that different reactions might be evoked in a single patient by different apple cultivars and also separately for the peel and the pulp of a single cultivar. The cultivars were ranked according to their allergenicity level, being Jonathan, Jonagold, Golden Delicious and Fiesta the most allergenic when considering the pulp and Gala, Fiesta and Golden Delicious for the peel. Further investigations are needed to clarify if a single patient can be allergic only to well-defined apple cultivars and which allergometric tests are necessary to ascertain this.

Keywords Apple allergy, apple cultivars, food allergy, Mal d 1, Mal d 3, prick-to-prick skin prick test, recombinant allergens.

Introduction

Plant foods are the most common cause of allergy among children over 5 years of age and young adults, and Rosaceae fruits are the most frequently involved (Eriksson *et al.*, 1982; Ortolani *et al.*, 1988; Cuesta Herranz *et al.*, 2000; Kanny *et al.*, 2001; Fernández-Rivas *et al.*, 2008). A recent study based on patient-reported data estimated a perceived fruit allergy in 2.2%–11.5% of children aged 0–6 and in 0.4%–6.6% of adults worldwide (Zuidmeer *et al.*, 2008). Allergic reactions against these fruits are often associated with pollinosis (Eriksson, 1978), because of IgE cross-reactivity to homologous proteins. However, they can also occur without related pollinosis (Fernández-Rivas *et al.*, 1997, 2006). Among Rosaceae, the prevalence of apple allergy is noteworthy, and the cross-reactivity of Mal d 1 with the main birch pollen allergen Bet v 1 has already been described in many studies (Breiteneder *et al.*, 1989; Hoffmann-Som-

mergruber *et al.*, 1996; Nucera *et al.*, 2009), as well as the other apple allergens Mal d 2–4 (Marzban *et al.*, 2005). These proteins are known to induce different symptoms in allergic patients: Mal d 1 usually causes symptoms of the oral allergy syndrome (OAS), because of cross-reactivity to Bet v 1 (Ricci *et al.*, 2005; Matthes & Schmitz-Eiberger, 2009); Mal d 3, a lipid-transfer protein (LTP), is associated with systemic reactions in up to one-third of the cases (Fernández-Rivas *et al.*, 2006). Symptoms correlated to Mal d 2 and Mal d 4 are not completely clarified. Several allergy patterns have previously been described involving the different apple allergens (Andersen *et al.*, 2009).

A different allergenicity level has been described depending on the apple cultivar (Bolhaar *et al.*, 2005). Furthermore, LTP seems to be particularly localised on fruit peel (Borges *et al.*, 2006), suggesting a different allergen expression between apple pulp and peel.

The aim of this research was

- to evaluate differences in skin prick test (SPT) response to apple cultivars in an Italian population of patients with apple allergy;

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- to identify a relationship between sensitisation to the apple recombinant allergens (Mal d 1 and Mal d 3) and symptoms;
- to verify the cross-reactivity between apple recombinant allergens and pollen-specific IgE.

Materials and methods

Nineteen patients with a history of apple allergy were recruited from the Pediatric Allergology Outpatient Clinic of the University of Bologna, 10 of which were women (53%) and 9 were men (47%), median age = 15 and IQR = 9–21 years. Inclusion criteria were one or more reactions after eating apples, either local (OAS) or systemic (gastrointestinal symptoms, widespread dermatitis, urticaria, anaphylaxis), in patients with or without a history of respiratory allergies and in the absence of systemic or chronic illnesses apart from asthma, allergic rhinoconjunctivitis and atopic dermatitis.

Among the 19 subjects of the study, 9 (47%) were 13 years old or younger and were considered as children. No statistically significant (s.s.) difference for gender was highlighted between adults and children (Fisher exact test $P = 0.179$).

All patients were evaluated by means of SPTs performed on the flexor surface of the forearm by using the prick-to-prick (PTP) technique, using the peel and the pulp of the apple separately. All patients were also tested with the commercial apple extract (Lofarma). For interpatient comparability, results were compared to the response to the positive control (histamine dihydrochloride) and were considered as positive (+1) when the wheal diameter was at least the same as histamine or larger, and as negative (0) when the wheal diameter was lower than that of histamine.

Eleven different commercial (Fiesta, Florina, Fuji, Gala, Golden Delicious, Jonagold, Jonathan, Red Chief, Topaz) and noncommercial (Durello, Ohrin) apple cultivars were chosen for an assessment of their allergenicity and were tested using the PTP method. The fruits were stored in a cold room at 4 °C. Eleven patients of nineteen were tested with all of the apple cultivars genotypes, whereas eight of them were only tested with ten.

The same apple test was not repeated in the same subject; nevertheless, SPTs were performed by the same operator and in a few months time range to reduce the possible intra-assay variability. The same apple, however, was used for testing in different patients only during the same day; if an apple had already been cut for testing, it was not used on the following days and a different apple was cut to test other patients in other days. The apples were constantly stored in the fridge to minimise the possible changes in their allergenic characteristics over time.

Apple-specific IgE, Mal d 1-specific IgE and Mal d 3-specific IgE (sIgE) were also determined in all patients (Unicap 1000; Phadia, Uppsala, Sweden). The procedure for evaluating sIgE for Mal d 1 and Mal d 3 includes the testing of the negative control serum also after its incubation with the serum of a nonapple-allergic subject but with elevated total serum IgE (1000 KU L⁻¹). This allows verifying the absence of an interference of total IgEs on the results of the test, thus avoiding the risk of false positive results. Even after this workup, the testing of the control serum resulted as negative.

Informed consent was obtained from the patients and/or their caregivers. The study was approved by the Local Ethical Committee (protocol n. 121/2008, approval date 16/12/2008).

Statistical analyses were carried out after standardisation of the diameter of the wheal determined by the tested apple or extract and the diameter of the wheal induced by histamine. This means that a value of one indicates a result corresponding to that of histamine, a value < 1 a less severe reaction than to histamine and a value over one a more severe reaction than to histamine.

Statistical analysis was performed by SPSS 17.0 for Windows.

Clinical data rarely have a gaussian distribution and in medicine percentiles are often preferred for descriptive statistics in skewed distributions (when mean may be unreliable).

If a set of clinical measures has n elements and is arranged in increasing order: $\chi_1 \leq \chi_2 \leq \dots \leq \chi_n$, median or 50th percentile (a type of average) is the numeric value that splits the higher half of the set of measures from the lower one.

Interquartile range (IQR) is used as a measure of statistical dispersion, being equal to the difference between the 75th percentile (the numeric value that cuts off lowest 75% of data) and the 25th percentile (the numeric value that cuts off lowest 25% of data). It is also called the midspread or middle fifty and this value is important when two or more sets of data are compared. To evaluate the heterogeneity in symptoms of our sample, Gini coefficient (ranging from 1, maximal heterogeneity, to 0, maximal homogeneity) was performed (Gini, 1921; Dixon *et al.*, 1987).

Owing to the skewed distribution of measures, percentiles and nonparametric tests were chosen either to compare them in the different cultivars and between adults and children (Mann–Whitney test), or to identify differences in allergic reaction to the pulp and the peel of the same cultivar (Wilcoxon test, Friedman chi square) or to evidence correlations between the different allergometric parameters (Kendall τ). Fisher exact test was used for nominal data. A 1st type error $P \leq 0.05$ was accepted.

To graphically display data, box and whisker plots (Tukey, 1977) were preferred to histograms because they are useful to display differences between populations without making any assumptions of the underlying statistical distribution. The spacings between the upper and lower limits of the boxes indicate the degree of dispersion (spread between the 25th percentile and the 75th percentile), and skewness in the data is evidenced by the asymmetric position of 50th percentile in the box; whiskers indicate the spread between the 10th and the 90th percentile and by this method it is possible to identify the number and the value of outliers that are represented as dark dots (extremely outliers are represented as little stars), having a summarised but complete view of raw standardised data.

Results

Fifteen of the 19 patients were affected by OAS, 2 patients referred to a previous glottal oedema (one of them with OAS) and 2 had only gastrointestinal symptoms (modal frequency: OAS 78.85%, Gini coefficient = 0.68). Eighteen patients (94.7%) had positive sIgE to *Betula verrucosa*. Figure 1 shows patients' levels of serum sIgE to apple, Mal d 1, Mal d 3 and the most frequent inhalant and food allergens.

Table 1 shows the results of sIgE for Mal d 1 and Mal d 3 of the study patients, of the negative control and of the same negative control after incubation with a serum with IgE 1000 kU/l.

Commercial standardised SPT wheals were medianly 0.75 (IQR 0.33-1). Table 2 shows the differences in allergenicity of the pulp and the peel for the different cultivars, examined by PTP-SPT. Apparently, the pulp seems more allergenic than the peel, for Fuji, Jonagold and Golden Delicious, in this patient population, while the peel is more allergenic than the pulp for Florina, Fiesta, Ohrin and Gala, but only for Gala the difference is s.s.

Some differences were noticed also comparing the wheals obtained by SPT with the commercial extract and PTP-SPT of all the cultivars both for peel and pulp. In all but three apple cultivars (Florina, Durello, Fuji), the SPT with the commercial extract gave s.s. smaller wheals than those caused by PTP-SPT with the peel and pulp of the apples (Table 2).

Figs 2 and 3 show the ranking of the different apple cultivars after standardisation and according to their allergenicity, separately for the pulp and the peel.

Apple sIgE show an s.s. correlation with Mal d 3 sIgE (Kendall Tau = 0.465 with $P < 0.006$), whereas there is no correlation between apple sIgE and Mal d 1 sIgE (Kendall Tau = 0.188 with $P = 0.263$).

We also analysed the correlations between the wheals induced by the peel and the pulp of the different cultivars and SPT with the commercial extract, Mal d 1, Mal d 3 and apple sIgE. However, the results of this test did not identify any strong relationship between the included parameters. In particular, there was no s.s. correlation with Mal d 3 and apple sIgE; only one

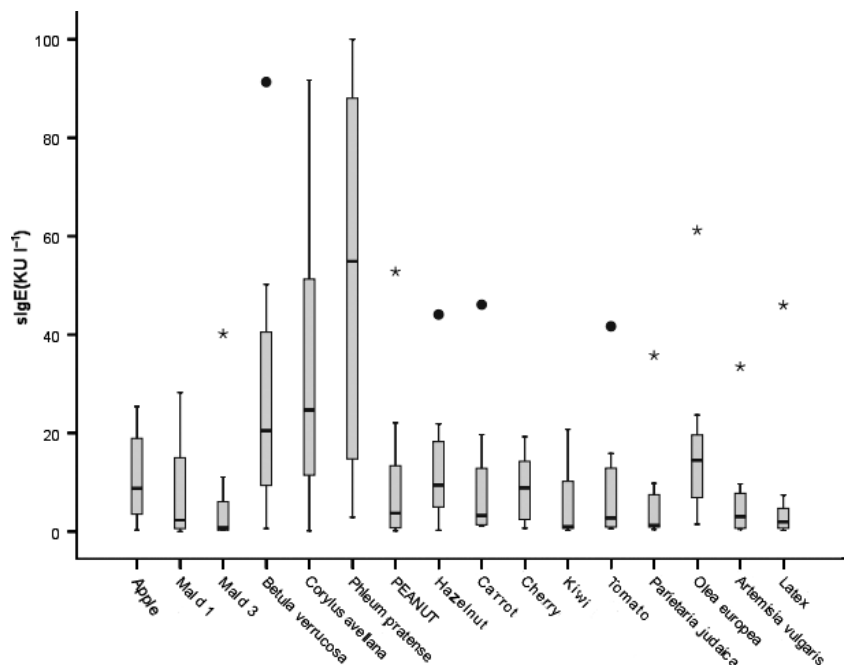


Figure 1 Patients' levels of serum sIgE to apple, Mal d 1, Mal d 3 and the most frequent inhalant and food allergens. The dark dots represent the outliers and the little stars the extremely outliers values.

Table 1 Values of sIgE for Mal d 1 and Mal d 3 of the study patients. Results of the negative control and the negative control added with a serum with IgE 1000 kU/l are shown

Serum No.	Mal d 1	Mal d 3
1	4.63	0.26
2	12.31	2.46
3	10.23	0.23
4	1.20	1.07
5	0.05	0.36
6	23.65	29.38
7	22.75	0.27
8	3.06	0.19
9	7.29	0.77
10	56.73	4.64
11	10.09	0.23
12	2.42	0.30
13	16.84	27.40
14	28.26	11.09
15	32.01	0.32
16	0.09	0.27
17	2.33	40.18
18	0.06	6.24
19	0.09	0.31
neg.	0.06	0.38
neg + 1000	0.12	0.53

positive correlation between PTP with Red Chief peel and SPT with the commercial extract (Kendall Tau = 0.366, $P < 0.05$); some positive correlations between Mal d 1 sIgE and PTP with Florina peel (Kendall Tau = 0.375, $P < 0.05$), Red Chief pulp (Kendall Tau = 0.450, $P < 0.01$), Jonathan peel (Kendall Tau = 0.362, $P < 0.05$), Topaz pulp (Kendall Tau = 0.457, $P < 0.01$), Fiesta pulp (Kendall Tau = 0.511, $P < 0.01$), Fuji peel (Kendall Tau = 0.366, $P < 0.05$)

and pulp (Kendall Tau = 0.416, $P < 0.05$), Gala peel (Kendall Tau = 0.451, $P < 0.01$) and pulp (Kendall Tau = 0.358, $P < 0.05$), and Golden Delicious peel (Kendall Tau = 0.503, $P < 0.01$).

No s.s. correlations were identified between symptoms and sensitisation to Mal d 1, Mal d 3, apple-sIgE and the cutaneous reactions to the different cultivars.

An s.s. correlation was identified between sIgE for *Betula verrucosa* and Mal d 1 ($P = 0.004$) but not for Mal d 3.

Children never developed larger wheals than adults. In five cultivars (Table 3), the adult patients developed s.s. larger wheals with PTP-SPT than those of children for Fiesta pulp and peel, Gala pulp and peel, and Jonathan peel.

Discussion

Our results confirm the fact that SPT with the commercially available apple extract does not seem to be a valid instrument to perform allergometric tests in patients with a history of a possible allergic reaction to apple. It is well known that Mal d 1 is a labile allergen and, although several techniques have been proposed to avoid breakdown and improve the quality of the apple extracts (Bjorksten *et al.*, 1980; Vieths *et al.*, 1994, 1998), even small differences in its extraction can have a very significant effect on its reactivity (Bolhaar *et al.*, 2005).

It is thus necessary to clearly define the role of the different tests that should be performed in the case of a clinical suspect of IgE-mediated reactions, not to underestimate an apple allergy, because a further ingestion might induce more severe symptoms.

Table 2 Percentiles of the wheal diameters obtained after prick-to-prick (PTP)- skin prick test (SPT) (standardised over histamine) of commercial extract and apple cultivars, separately tested with the pulp and the peel

PTP-SPT wheals compared with the commercial extract					
Cultivar	Peel Median (25thP–75thP)	Pulp Median (25thP–75thP)	Commercial extract Median (25thP–75thP)	Friedman chi square	
Durello	1.3333 (0.2500–1.0000)	1.0000 (0.500–1.0000)	0.7500 (0.3333–1.0000)		n.s.
Fiesta	1.3333 (1.0000–2.0000)	1.1667 (0.8333–1.5000)		11.841	$P \leq 0.003$
Florina	1.3333 (0.5000–2.0000)	1.0000 (0.6667–1.6667)			n.s.
Fuji	1.2500 (0.2500–2.0000)	1.3333 (0.3333–2.0000)			n.s.
Gala*	1.3333 (1.0000–1.6667)	1.0000 (1.0000–1.5000)		16.265	$P \leq 0.0001$
Golden delicious	1.3333 (1.0000–1.6667)	1.2500 (0.6667–1.5000)		10.239	$P \leq 0.006$
Jonagold	1.0000 (0.2500–1.3333)	1.2500 (1.0000–1.7500)		13.059	$P \leq 0.001$
Jonathan	1.0000 (0.6667–1.5000)	1.0000 (0.8333–2.0000)		13.373	$P \leq 0.001$
Ohrin	1.2500 (1.0000–1.6667)	1.0000 (0.6667–1.2500)		10.551	$P \leq 0.005$
Red Chief	1.0000 (0.6667–1.6667)	1.0000 (0.6667–1.2500)		14.597	$P \leq 0.001$
Topaz	1.0000 (0.6667–1.3333)	1.0000 (0.6667–1.6667)		13.373	$P \leq 0.001$

*Peel > pulp PTP-STP in 11 patients for Gala cultivar, Wilcoxon test: $z = -1.999$, $P < 0.05$

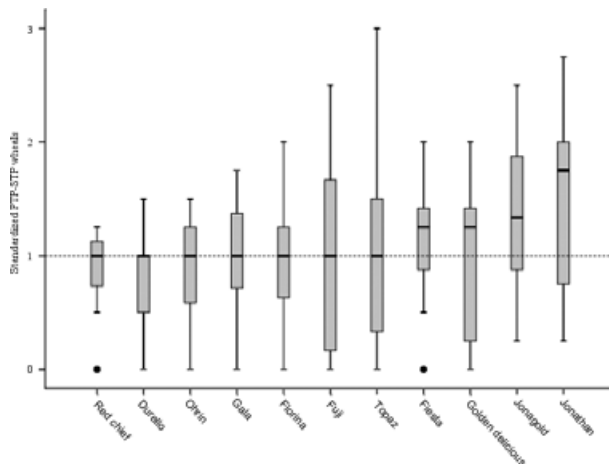


Figure 2 Ranking of the different apple cultivars after standardisation and according to their pulp allergenicity. The dark dots represent the outliers.

When considering the peel and the pulp separately, a different allergenicity of the apple cultivars was apparently highlighted, as shown in Table 2. For Fuji, Jonagold and Golden Delicious, the pulp seems to be more allergenic than the peel, and for Florina, Fiesta, Ohrin and Gala, the peel seems to be more allergenic than the pulp. However, the difference is s.s. only for Gala. Fernández-Rivas *et al.* highlighted that, in Spain and to a lesser extent in Italy, skin reactivity to the peel was higher than to the pulp (Fernández-Rivas *et al.*, 2006). Also previous reports suggested a higher allergenicity of the peels of Rosaceae fruits than their pulps (Fernández-Rivas & Cuevas, 1999). This might be of interest for future studies that wish to understand the individual patients' allergic profiles.

A ranking in the allergenicity of the different apple cultivars is described in Figs 2 and 3, separately considering the pulp and the peel. For what concerns the pulp, Jonathan, Jonagold, Golden Delicious and Fiesta appeared to be the most allergenic apple cultivars. As for the peel, Gala, Fiesta and Golden Delicious apparently caused the larger wheals in our population. It is interesting to stress that in our study Gala, whose peel is the most allergenic, was among the least allergenic cultivars when the pulp was considered. On the other hand, Jonathan is the cultivar that caused the larger wheal reactions with its pulp but is not very allergenic when the peel is considered. Moreover, it apparently stands out that a single apple can be among the most allergenic with its pulp or peel and among the least allergenic when considering the other part.

Our results seem to evidence that sensitisation to Mal d 3 correlates with apple sIgE but does not correlate

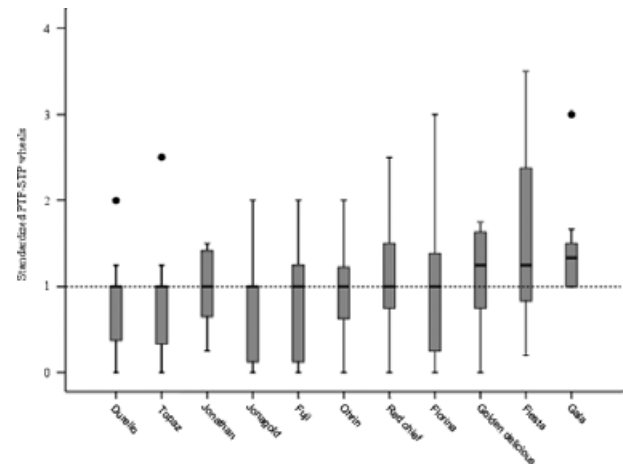


Figure 3 Ranking of the different apple cultivars after standardisation and according to their peel allergenicity. The dark dots represent the outliers.

with the results of the PTP-SPT with any of the examined apple cultivars; on the contrary, sensitisation to Mal d 1 does not correlate with apple sIgE but seems to correlate with several apple cultivars as measured by the reaction to PTP-SPT. However, in our study neither Mal d 1 nor Mal d 3 sIgE correlated with the clinical severity of the reactions to apple ingestion. In a study by Fernández-Rivas *et al.*, the IgE responses to Mal d 1 and Mal d 3 were associated with the clinical presentation of apple allergy, being sensitisation to Mal d 3 a risk factor for systemic reactions (Fernández-Rivas *et al.*, 2006). These results might indicate a possible role of the recombinant allergens in the evaluation of patients with apple allergy. The fact that our data do not show any relationship between symptoms and sensitisation to recombinant allergens might be because of the small number of patients that were included in the study and to the very important prevalence of OAS in a sample that was enough homogeneous. A recent research by Gao and colleagues analysed the role of the allelic variants of Mal d 1 and highlighted the importance of qualitative and quantitative aspects of Mal d 1 gene expression on allergenicity (Gao *et al.*, 2008).

Our results also seem to confirm the well-known relationship between birch and apple allergy. It is well known that, because of cross-reactivity between Bet v 1 and proteins present in several plant-derived foods, birch pollen allergic patients develop food allergies, most frequently to apples (Matthes & Schmitz-Eiberger, 2009). Bet v 1, the major apple allergen of birch, shares a high degree of structural homology with Mal d 1, being both allergens of the PR-10 family (Andersen *et al.*, 2009). On the contrary, there is no relationship between sIgE for *Betula verrucosa* and Mal d 3: in fact, the latter

Table 3 Allergens that caused s.s. larger standardised wheals in adults compared to children (data standardised over histamine)

		PTP-SPT wheals Compared between Adults and Children		
Cultivar allergens		Children (<i>n</i> = 8) Median (25thP–75thP)	Adults (<i>n</i> = 11) Median (25thP–75thP)	Mann–Whitney <i>z</i>
Durello	Peel		0.6000 (0.3750–1.000)	1.0000 (0.1875–1.4375)
	Pulp		1.0000 (0–1.0833)	1.0000 (0.667–2.000)
Fiesta	Peel		1.0000 (0.3167–1.3125)	1.6667 (1.2500–2.7500)
	Pulp		1.0000 (0.1875–1.1250)	1.5000 (1.0000–1.6667)
Florina	Peel		0.8333 (0–1.650)	1.3333 (1.0000–2.2500)
	Pulp		0.9167 (0.6167–1.500)	1.0000 (0.6667–2.0000)
Fuji	Peel		0.6250 (0–1.5500)	1.3333 (0.7500–2.3333)
	Pulp		0.9750 (0.0833–1.3333)	1.7500 (0.3333–2.0000)
Gala	Peel		1.0000 (1.000–1.3000)	1.5000 (1.3333–1.6667)
	Pulp		0.9167 (0.3375–1.0000)	1.2500 (1.0000–1.5000)
Golden delicious	Peel		1.2500 (0.1250–1.6500)	1.3333 (1.0000–1.6667)
	Pulp		0.8333 (0.0625–1.3333)	1.3333 (1.0000–1.6667)
Jonagold	Peel		0.5000 (0–1.2500)	1.2500 (0.6667–1.6667)
	Pulp		1.0000 (0.77708–1.5833)	1.3333 (1.0000–2.0000)
Jonathan	Peel		0.9000 (0.2500–1.2500)	1.5000 (1.0000–2.0000)
	Pulp		1.7083 (0.7500–1.9500)	1.0000 (0.8333–2.0000)
Ohrin	Peel		1.1000 (0.2500–1.3333)	1.3333 (1.0000–2.0000)
	Pulp		0.9167 (0.1667–1.0000)	1.0000 (0.6667–1.5000)
Red Chief	Peel		1 (0.3750–1.3750)	1.5000 (1.0000–1.6667)
	Pulp		0.9000 (0.1667–1.1875)	1 (1.0000–1.2500)
Topaz	Peel		0.8750 (0.5417–1.2500)	1.2500 (1.0000–2.0000)
	Pulp		0.5000 (0–1.0000)	1 (1.0000–1.6667)

PTP, prick-to-prick; SPT, skin prick test.

does not share any common structure with the birch allergens, belonging to a different kind of protein family (LTP allergens).

In our study, adult patients had some s.s. larger wheal reactions to PTP-SPT than children. This was true for both the pulp and the peel of Fiesta and Gala, and only for the peel of Jonathan. To the best of our knowledge, there are no reports in the literature of a different skin reactivity among adults and children affected by apple allergy. The interest of this point might be related to a different clinical severity of the allergic reactions to apple in the youngest patients compared to adults. The difference in SPT reactivity between children and adults might also be partly because of the fact that children have less developed birch pollen allergies. However, a different skin reactivity at various ages could be a matter of further study.

Finally, Table 1 highlights that a few patients, despite having had an allergic reaction to apple ingestion, have negative sIgE to both Mal d 1 and Mal d 3. In these cases, different allergens are probably involved (possibly Mal d 2 and Mal d 4), but they could not be analysed in this study.

The major limitation of the present study is related to the low number of patients that are included. A larger

case series, including both adults and children, might have allowed a confirmation of some of the results obtained (role of recombinant allergens in the allergometric evaluation, possible differences in PTP-SPT reactions in different age groups).

Another important limitation is that the present study only included a limited number of apples and all coming from a single harvest. The tests were not repeated in different seasons, what might have been useful to identify further differences in allergenicity. Previous studies indicate a high variability content in Mal d 1 within the same cultivar cultivated in different orchards and also in different years, and it was found that Mal d 1 expression is influenced by several biotic and abiotic factors, such as cultivation methods, exposure to fungi, viruses, bacteria etc (Matthes & Schmitz-Eiberger, 2009). The exact mechanisms that lead to Mal d 1 expression, though, have yet to be investigated.

Despite so many limitations in the present study and the obscure points regarding apple allergenicity, it is important to highlight that different apple cultivars might evoke different reactions in the same patient. Previous studies showed different levels of Mal d 1 and Mal d 3 in different apple cultivars (Zuidmeer *et al.*, 2006; Sancho *et al.*, 2008; Matthes & Schmitz-Eiberger,

2009). Recently, component-resolved diagnosis has been proposed for performing SPT with purified allergen proteins (Asero *et al.*, 2008). A further investigation in this sense might clarify if a single patient can be allergic only to well-defined apple cultivars and which allergometric tests are necessary to ascertain this.

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