

SEARCH FOR LOW ALLERGENIC APPLE CULTIVARS FOR BIRCH POLLEN-ALLERGIC PATIENTS: IS THERE A CORRELATION BETWEEN *IN VITRO* ASSAYS AND PATIENT RESPONSE?

R. Asero (1), G. Marzban (2), A. Martinelli (3), M. Zaccarini (3),
M. Laimer da Câmara Machado (2)

SUMMARY:

Background: - Due to the cross-reactivity between Bet v 1 and proteins present in vegetable foods, birch pollen allergic patients frequently develop allergy to fruits and vegetables, mostly apples. Since many apple cultivars exist, some of them might contain sufficiently low amounts of Mal d 1 to be tolerated by most allergic patients.

Objective: To assess whether apple cultivars containing low amounts of Mal d 1 are better tolerated by apple-allergic patients.

Methods: - Mal d 1 content was determined in many apple cultivars by ELISA. Selected cultivars containing high (Golden Delicious) or low (Orin, G 198 and Vienna) amounts of Mal d 1 were compared in apple allergic patients both by SPT and oral challenges.

Results: The 3 different apple cultivars induced wheals of similar size in most patients. Upon oral challenges no patient reported the total absence of oral symptoms following the ingestion of either high or low allergenic apples. Golden Delicious and G-198 elicited OAS of similar severity 3/7 cases. The 2 cultivars induced significantly more severe symptoms in 2 cases each.

Conclusion: Allergy to Mal d 1 is characterized by significant inter-patient variability. Moreover, marked inter-apple and intra-apple variability exists. As a consequence, the amount of Mal d 1 in apples classified as containing low concentrations of allergen may be sufficient to induce both clinical symptoms and skin reactivity in birch pollen-allergic patients. The search for low allergenic apples therefore should be continued and extended to other germplasm accessions, be it cultivars, breeding lines or wild species.

Key-words: Food allergy - Apple - Mal d 1 - Birch - Bet v 1.

INTRODUCTION

About 98% of birch pollen allergic patients are sensitised to the major allergen, Bet v 1 (1), a highly conserved pathogenesis-related protein class 10. Due to the cross-reactivity between Bet v 1 and homologue proteins present in a number of plant-derived foods a large proportion of birch pollen allergic patients also develop food allergy to an array of fruits and vegetables (2, 3); apple represents the most frequently involved food in these subjects (2-4). Bet v 1-homologue apple proteins are rather labile and sensitive to heat, pepsin digestion and oxidation (5, 6); as a consequence symptoms induced by foods are generally slight, often limited to immediate

itching of the lips and oral mucosa following the ingestion of the fresh, raw offending fruits and vegetables (the so-called oral allergy syndrome [7]); nonetheless, cases of laryngeal oedema have been sometimes reported (8). Allergy to apples can be induced by three further allergens: Mal d 2, a thaumatin-like protein (TLP) of 31 kDa, Mal d 3, a 9 kDa non-specific lipid transfer protein (LTP), with high resistance to peptidase and heat, and Mal d 4, the panallergen profilin of approx. 14 kDa (9, 10). The different types of apple allergy show a typical geographic distribution, with Mal d 1 allergy that is virtually absent in areas where birch pollen is not present and LTP (Mal d 3) allergy that has been mostly reported from Mediterranean countries. The concentration of Mal d 1 may show much variability from one apple cultivar to another (11, 12). Since a number of apple cultivars are available throughout the world, it might be possible that in some of them amounts of Mal d 1 are sufficiently low to guarantee good tolerance by most

(1) Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano (MI) (Italy).

(2) Plant Biotechnology Unit, IAM, Dept. Biotechnology, BOKU, Vienna (Austria).

(3) Consorzio Italiano Vivaisti, Ferrara (Italy).

apple-allergic patients. Indeed there are reports of apple allergic patients tolerating one or the other cultivar. The individual kind of patient reactivity can be an additional factor justifying tolerance to apples with different amounts of Mal d 1. In the present study the Mal d 1 concentration range was determined in a number of apple cultivars and the effects of selected cultivars containing high or low amounts of Mal d 1 were compared *in-vivo* in apple allergic patients.

PATIENTS AND METHODS

Apple cultivar screening

Apples from an experimental field on sandy soils near the Adriatic seaside with drip irrigation systems were harvested at cultivar specific dates, and stored for three months at 4°C before extraction. Integrated plant protection was carried out according to Reg. CEE 2078, including herbicide treatment within the rows. Clearly, to assure a valid ranking, the sampling procedure had to be adapted. Initially, to determine the intra and inter apple variability for Mal d 1 in different cultivars, five apples of different cultivars were cut into quarters, and each piece separately extracted and analysed for its Mal d 1 content by ELISA. For this work we have selected two cultivars, Golden Delicious and Jonagold, which are classified as high and low allergenic in cultivar ranking (12). As a consequence from the obtained data, we considered the following procedure as reliable for the purpose of ranking of the Mal d 1 content of different cultivars: five apples per cultivar were peeled (5 mm) with a pairing machine, shock frozen and powdered in liquid nitrogen before extraction as described (12). Concentration of total protein was determined with the BCA protein assay kit (PIERCE, U.S.A) according to manufacturer's instructions. All extracts were buffer-exchanged into PBS using PD-10 columns (Amersham Biotech, U.S.A.) before protein determination due to high contents of reducing agents in apple tissue. Eight apple cultivars, including currently commercialised (Golden Delicious, Jonagold, Fuji, Rubens, Granny Smith), locally grown old cultivars (Vienna) and new breeding lines (Orim, G 198) which still did not enter the market place, were compared for their Mal d 1 content ($\mu\text{g/g}$ fresh weight) using ELISA as previously described (12). Briefly, microtiter plates (Nunc-Immuno Plate MaxiSorp™ Surface) were coated with polyclonal rabbit anti-Mal d 1. For the detection a murine anti-Mal d 1 mAb 4C3C10 and alkaline phosphatase-labelled goat anti-mouse IgG (Fab specific; Sigma A1682) were used. Apple extracts and antibodies were diluted in TBS + 2% PVP + 0.5% BSA +

0,1% Tween20. Photometric measurements were performed at 405 nm (reference wavelength 620 nm) with a microplate reader (SLT). Absorbance values were expressed as ng Mal d 1 per ml extract by interpolation with the reference curve using the software DeltaSoft from BioMetallics (based on the 4-parameter log/logit fit regression analysis). All determinations were run in duplicates and Mal d 1 amounts were converted to μg Mal d 1/g fresh fruit and as % of Mal d 1 of total soluble protein.

Obtained data offered an insight into allergen levels of eight different apple cultivars - ranging between 0.84 - 33.17 $\mu\text{g/g}$ fresh weight. Cultivars could be divided into three groups containing high, medium or low amounts of Mal d 1; cultivars known as highly allergenic from clinical practice (e.g. Granny Smith or Golden Delicious) were finally found in the high ranking group and Orim and G198 in the low group. Previous studies showed that the Mal d 1 is equally expressed both in peel and in pulp of all cultivars tested (10). Based on data obtained in the first cultivar screening carried out in 2003 Orim, G 198, and Vienna apples were chosen as low allergenic apples for a comparison with the high allergenic Golden Delicious on allergic patients (table 1). Apples of the 2004 harvest were handled in separate batches for the measurement of Mal d 1 content and for SPT experiments in 2004.

Patients

Seventeen consecutive adult patients (M/F 8/9; mean age 33 years, range 26-49 years) with a history of oral allergy syndrome (defined as oral itching with or without angioedema of the lips and/or tongue) induced by apple seen during the second half of 2004 were studied. All were diagnosed as being monosensitized to birch pollen on the basis of a clinical history of seasonal rhino-conjunctivitis with or without asthma from the beginning of March to mid May and of a strongly positive SPT with a commercial birch pollen extract (50000 SBU/ml; Allergopharma, Reinbeck, Germany) in the absence of skin reactivity to grass, mugwort, ragweed, pellitory, plantain, olive, and cypress pollen (Allergopharma) as well as to latex proteins (0.5 mg/ml; Abello, Lainate, Italy). This excluded sensitisation to apple profilin (10). Hypersensitivity to lipid transfer protein was excluded by negative SPT with a commercial peach extract (5% w/v; Abello) as previously observed (13, 14).

No patients had undergone specific immunotherapy with birch pollen extract before, and no patient was taking anti-histamines or corticosteroids when SPT and oral challenges were carried out.

Skin tests

Based on the 2003 apple cultivar ranking (see above) all study patients underwent SPT with one fresh "high allergen content" apple (Golden Delicious) and 2 "low allergen content" apples (G-198 and Orim in 9 patients; Orim and Vienna in 8 patients). G 198 and Orim were breeding lines provided together with Golden Delicious by CIV (Consorzio Italiano Vivaisti, Ferrara, Italy). The Vienna apple was provided by IAM, BOKU, Plant Biotechnology Unit, Vienna, Austria, following the observation that this old local cultivar could be ingested without disturbance by an Austrian patient that had otherwise apple allergy as confirmed by SPT and oral challenges with Golden Delicious.

All SPT were carried out in duplicate by the prick-prick technique using the fresh apples and read following established methods (15); a mean wheal diameter > 3mm was considered positive. SPT with saline and histamine 10 mg/ml were carried out as negative and positive controls, respectively.

Oral challenges

Seven patients accepted to undergo single blind comparative oral challenges with one "high allergen content" apple (Golden Delicious) and one "low allergen content" apple (G-198 in 6 cases; Orim in 1 case). The Vienna apple was not employed for oral challenges due to its poor storage capacity; oral challenges with this cultivar are scheduled for the next season. In order to avoid recognition of the challenged cultivar, oral challenges were carried out using only freshly cut apple pulp (without peel); previous studies showed that Mal d 1 concentration does not differ in apple peel and pulp (12). Patients were asked

to chew about 15 g of apple for about 3 minutes before swallowing it. After 15 minutes patients were asked to report whether the fruit induced any oral symptom and, if so, to grade the severity on a visual analogue scale ranging from 0 to 10. Challenges with the two distinct apple cultivars were carried out at least 2 days apart in a randomised sequence.

RESULTS

Results obtained from the determination of inter- and intra-apple variability of the Mal d 1 content of different cultivars indicate a ubiquitary, though uneven distribution of this allergen in different cultivars (figure 1; ref 12). With these data we could demonstrate the relative variability of Mal d 1 content in five individual apples of the same cultivar, and within four pieces of each single apple. This has led to an adap-

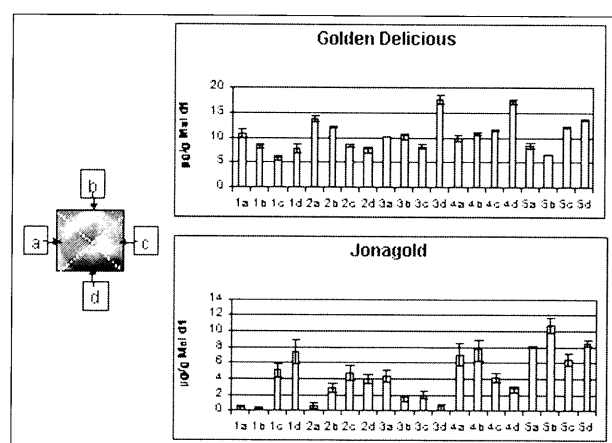


Figure 1: To monitor the inter- and intra-apple variability for Mal d 1 content, five apples (1-5) of cultivars Golden Delicious and Jonagold were cut into quarters (a, b, c, d), extracted and analysed by ELISA. All determinations were run in duplicates, and calculated as mean values. The bars indicate the standard deviations.

Apple cultivar	I. Mal d 1 content µg/g	I. Total Protein µg/g	II. Mal d 1 content µg/g	II. Total Protein µg/g	% Mal d 1 /total soluble protein	III. Mal d 1 content	III. Total Protein µg/g	% Mal d 1 /total µg/g soluble protein
Golden Delicious	5.6	n.d.	12.18	1675.5	0.78	5.5	1281.2	0.42
Granny Smith	5.45	n.d.	12.14	1200.3	1.01	-	-	-
Fuji	5.4	n.d.	11.5	1826.9	0.24	-	-	-
Rubens	6.72	n.d.	7.23	1749.3	0.38	-	-	-
Jonagold	5.05	n.d.	3.33	1742.4	0.19	-	-	-
Vienna	2.98	n.d.	2.45	1212.0	0.20	3.79	2400.0	0.16
G198	2.94	n.d.	3.47	2037.5	0.17	4.7	1364.3	0.34
Orim	1.05	n.d.	0.84	2080.5	0.04	2.62	1881.0	0.14

Table 1: Serial measurements of Mal d 1 content in different apple cultivars. Mal d 1 values are expressed in µg/g fresh weight, and in % of total soluble protein. I: Mal d 1 content of different cultivars was measured for the first time in 2003. II: Measurements were repeated from a new lot of the same cultivars in the same year 2003. III: Results obtained for the cultivars used for clinical trials in 2004. n.d.: not determined.

tation of the extraction procedure for standard extraction (12). The results obtained showed that the calculated Mal d 1 content is a normalized value, not valid for each individual apple of the same cultivar or for all tissues of a single apple. Additionally the rate of the variability is different for various cultivars, different years (table 1), and also production systems (12). Jonagold showed a higher rate of intra-(up to 20 fold) and inter-apple variability (up to 5 fold) than of Golden Delicious (2 fold).

The new apple cultivar screening showed that the differences in Mal d 1 content between the cultivars employed for this study results were much less evident in 2004 than in 2003 (table 1). The marked variability of batches from different years was further confirmed in a shelf life experiment carried out with a few cultivars in 2003/2004 (Marzban et al., unpublished).

Results of SPT and of oral challenge tests with different apple cultivars are summarized in table 2.

Apples belonging to the high or low Mal d 1 group induced wheals of similar size in most patients, though with a slightly reduced wheal in the case of

"low" candidates. One patient did not react in the SPT with all apple cultivars (table 2). On oral challenges none of the challenged patients reported the total absence of oral symptoms following the ingestion of one of the two apple cultivars. Golden Delicious induced more severe oral allergy syndrome in 2 cases (patients no. 4 and 9, table 2). The severity of oral symptoms elicited by Golden Delicious and by the low allergen apple was identical in 3/7 cases. G-198 induced a more severe oral allergy syndrome than Golden Delicious in 2 cases (patients no.6 and 7, table 2).

DISCUSSION

The impressive increase in the prevalence of respiratory allergies in Western countries that occurred during the last decades was paralleled by an increase in vegetable food allergy following primary birch pollen sensitisation. As a consequence, plant-derived foods currently represent by far the most frequent cause of food allergy in adults. The importance of Mal d 1 for real food allergy in Northern and of Mal d 3 in Central European countries has been demonstrated (16-18). In the present study we investigated whether the differences in Mal d 1 content between distinct apple cultivars could translate into a good tolerance of cultivars containing the lowest concentrations of this allergen in a group of apple-allergic patients monosensitized to birch pollen. Cultivars presumed to contain high (Golden Delicious) or low (Orim, G198, Vienna) amounts of Mal d 1 to be tested in allergic patients were selected on the basis of the ranking in a large apple cultivar screening carried out in 2003 (10). Surprisingly enough, the comparative in-vivo tests carried out in 2004 did not show any relevant difference in skin reactivity or in tolerance upon oral challenge between these apple cultivars. In keeping with in-vivo findings, new Mal d 1 measurements carried out on the same cultivars found that, in 2004, the concentrations of the allergen did no longer correspond to those recorded in 2003 and differences between Golden Delicious and the "low allergen content" cultivars were much less pronounced. Nonetheless, Mal d 1 concentrations as low as 2.6 µg/g fresh fruit (Orim cultivar) were able to elicit both significant skin reactivity and significant oral allergy syndrome in some Bet v 1-sensitized patients. One must consider that study patients were certainly hypersensitive to Mal d 1 and not sensitised to Mal d 4 (profilin) and Mal d 3, whereas possible sensitisation to Mal d 2 was not ruled out. However, Mal d 2 sensitisation seems rather unusual in Italian patients; in the recent EU-funded SAFE study (18) only 6/98 apple allergic Italian patients showed significant IgE

Patient -----SPT-----Challenge-----
Golden D G-198 Orim Wien Golden D G-198

1	8.5	8	8		
2	8	5	9	2/10	2/10
3	8	7	7	2/10	2/10
4	7.5	6	6.5	6/10	3/10
5	6.5	6.5	5		
6	9	10	9	2/10	5/10
7	6	8.5	4.5	2/10	8/10
8	14	14	11		
9	11	10	5	8/10	5/10
10	5		4.5	5	
11	4		4.5	5	
12	4		6	3	
13	Neg		Neg	Neg	
14	5		5.5	5	
15	5.5		4	3	
16	4		3.5	3.5	
17	8		8.5	6	4/10 4/10 (Orim)

Table 2: Results of SPT and oral challenges with different apple cultivars. SPT are expressed as mean wheal diameter. Neg: negative. Challenges are expressed as subjective severity of oral symptoms where 0 corresponded to absence of any symptom and 10 corresponded to extremely severe symptoms. (Orim): this patient was challenged with the cultivar Orim instead of G 198.

levels to rMal d 2. In the same series 91 patients were strong Mal d 1 reactors. As a consequence it seems rather unlikely that possible Mal d 2 sensitisation may have heavily influenced the results of the present study. The high variability in Mal d 1 content of single apple cultivars in different years and following storage for 3 weeks at 20°C RT (table 1) strongly suggests that even cultivars considered as containing reduced amounts of Mal d 1 may on some occasions show allergen concentrations that are not significantly different from those found in cultivars classified as highly allergenic (see figure 1) and, hence, high enough to elicit both significant skin reactivity and clinical symptoms in allergic patients.

Although further studies based on breeding approaches and long-term serial measurement of Mal d 1 are needed to definitively clarify whether some specific cultivars remain under a certain concentration year after year, the marked intra-cultivar variation in Mal d 1 content observed in this study suggests that the search for apples constantly expressing sufficiently low levels of Mal d 1 to be tolerated by most allergic patients might be a difficult task and therefore should be continued and extended to other germplasm accessions be it cultivars, breeding lines or wild species.

This study was performed within the SAFE project funded by the European Commission: QLC-CT-2000-01394.

References

1. Ebner C., Birkner T., Valenta R., Rumpold H., Breitenbach M., Scheiner O., Kraft D. - Common epitopes of birch pollen and apples. Studies by Western and Northern blot. *J Allergy Clin Immunol* 1991;88:588-94.
2. Eriksson N., Formgren H., Svenonius E. - Food hypersensitivity in patients with pollen allergy. *Allergy* 1982;37:437-43.
3. Dreborg S., Foucard T. - Allergy to apple, carrot and potato in children with birch pollen allergy. *Allergy*; 1983;38:167-72.
4. Lahti A., Bjorksten F., Hannuksela M. - Allergy to birch pollen and apple, and cross-reactivity of the allergens studied with the RAST. *Allergy* 1980;35:297-300.
5. Bjorksten F., Halmepuro L., Hannuksela M. - Extraction and properties of apple allergens. *Allergy* 1980;35:671-7.
6. Rudeschko O., Fahlbusch B., Henzgen M., Schlenvoigt G., Herrmann D., Vieths S., Jager C. - Investigation of the stability of apple allergen extracts. *Allergy* 1995;50:575-79.
7. Amlot P., Kemeny D.-M., Zachary C., Parkes P., Lessof M.-H. - Oral allergy syndrome (OAS) symptoms of IgE-mediated hypersensitivity to foods. *Clin Allergy* 1987;17:33-42.
8. Ortolani C., Pastorello E., Farioli L., Ispano M., Pravettoni V. et al. - IgE-mediated allergy from vegetable allergens. *Ann Allergy* 1993;71:470-5.
9. Breiteneder H., Radauer C. - A classification of plant food allergens. *J Allergy Clin Immunol* 2004;113:821-30.
10. Asero R. - Plant food allergies: a suggested approach to allergen-resolved diagnosis in the clinical practice by identifying easily available sensitisation markers. *Int Arch Allergy Immunol* 2005;138:1-11.
11. Vieths S., Jankiewicz A., Schoning B., Aulepp H. - Apple allergy: the IgE-binding potency of apple strains is related to the occurrence of the 18 kDa allergen. *Allergy* 1994;49:262-71.
12. Marzban G., Pühringer H., Dey R., Brynda S., Ma Y., Martinelli A., Zaccarini M., van der Weg E., Housley Z., Kolarich D., Altmann F., Laimer M. - Localisation and distribution of major apple allergens in fruit tissue. *Plant Science* 2005;169:387-394.
13. Asero R. - Detection and clinical characterization of patients with oral allergy syndrome caused by stable allergens in Rosaceae and nuts. *Ann Allergy Asthma Immunol* 1999;83:377-83.
14. Asero R., Mistrello G., Roncarolo D., de Vries S.-C. et al. - Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2000;122:20-32.
15. Dreborg S., Frew A. - Allergen standardization and skin tests. EAACI position paper. *Allergy* 1993; 48:49-75.
16. Schäfer T., Böhler E., Ruhdorfer S., Weigl L., Wessner D., Heinrich J., BFilipiak B., Wichmann H.-E., Ring J. - Epidemiology of food allergy/food intolerance in adults: associations with other manifestations of atopy. *Allergy* 2001;56:1172-1179.
17. Son D.-Y., Scheurer S., Hoffmann A., Haustein D., Vieths S. - Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *European Journal of Nutrition* 1999; 38:201-215.
18. Hoffmann-Sommergruber K. and the SAFE consortium. The SAFE project: "Plant food allergies: field to table strategies to reduce their incidence in Europe". An EC-funded study. *Allergy* 2005;60:436-42.

