

***In vivo* assessment with prick-to-prick testing and double-blind, placebo-controlled food challenge of allergenicity of apple cultivars**

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Background: Apple cultivars have been reported to differ in allergenicity on the basis of *in vitro* and skin prick tests with apple extracts.

Objectives: We sought to evaluate the efficacy of the prick-to-prick method in assessing differences in allergenicity of apple cultivars and to confirm differences by means of double-blind, placebo-controlled food challenge (DBPCFC).

Methods: Intra-assay and intracultivar variation of prick-to-prick test results were determined in 6 Dutch and 8 Spanish patients with apple allergy by using 5 apples of the cultivars Golden Delicious, Fuji, and Ecolette in duplicate. In addition, 21 cultivars were screened for allergenicity in 15 Dutch patients with birch pollen and apple allergy. Two selected cultivars (Golden Delicious and Santana) were tested with DBPCFCs. The influence of storage conditions on allergenicity was assessed in 5 cultivars.

Results: Intra-assay variation of skin prick testing was 3.9%, and intracultivar variation was 4.1%. A ranking of 21 cultivars was made on the basis of prick-to-prick tests in 9 patients. Apple cultivars were classified as of low, intermediate, and high allergenicity, with a significant difference between low and high allergenicity ($P < .001$). A significant difference in allergenicity determined between Golden Delicious and Santana cultivars ($P < .05$) was confirmed by means of DBPCFC. With 5 cultivars, controlled atmosphere (2.5% oxygen/1% carbon dioxide) was shown to reduce allergenicity ($P < .001$) by 15% compared with storage at 2°C.

Conclusions: Prick-to-prick testing with fresh apples is a reproducible method of assessing allergenicity. Apples can be classified as of low or high allergenicity for the majority of patients. This was confirmed by using DBPCFCs. Selection of

cultivars and control of storage conditions are both viable strategies for reduction of symptoms in patients with apple allergy. (J Allergy Clin Immunol 2005;116:1080-6.)

Key words: Allergy, hypoallergenicity, food, Bet v 1, Mal d 1, Mal d 3, double-blind, placebo-controlled food challenge

Apples are among the most common edible fruits and form an important source of vitamins and fibers in the diet of the European population. "An apple a day keeps the doctor away" is a common saying that originated with J. T. Stinson, the first director of the Missouri State Fruit Experimental Station in 1904 (T. Browne, personal communication). In Europe 20-25 kg per capita (ie, a total of >10 million metric tons) are consumed yearly, and the 6 most common cultivars on the market (79% market share) are Golden Delicious, Jonagold, Red Delicious, Gala, Elstar, and Granny Smith.¹

Unfortunately, up to 2% of the northern and central European population is allergic to apple.² Apple allergy in these parts of Europe is mainly encountered among patients with birch pollen allergy.^{3,4} This concordance is explained by cross-reactivity of IgE antibodies between birch pollen and apple allergens.^{5,6} The main cross-reactive birch pollen allergen is Bet v 1⁷; its homologue in apple is Mal d 1, which is homogeneously present in the peel and the pulp of the fruit.⁸ Mal d 1 is a proteolysis-sensitive allergen⁹ and causes predominantly mild and limited symptoms in the oral cavity (oral allergy syndrome).^{10,11} Furthermore, Mal d 1 does not survive most processing steps, like cooking or juice making, and thus only consumption of fresh apples leads to symptoms. Usually patients with apple allergy are also sensitized and (variably) allergic to other fruits, vegetables, and nuts on the basis of the same cross-reactive antibodies.^{6,12} In southern Europe apple allergy is less common and not related to birch pollen allergy.¹³ The clinical presentation of apple allergy is, however, more severe. This has been explained by the nature of the major apple allergen in the southern patients with apple allergy, the nonspecific lipid transfer protein (LTP) designated Mal d 3.¹⁴ LTP has been shown to be especially abundant in the peel of fruits.^{15,16}

A current management strategy for prevention of food allergy is based on the avoidance of consumption of

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Abbreviations used

DBPCFC: Double-blind, placebo-controlled food challenge
LTP: Lipid transfer protein
SPT: Skin prick test
VAS: Visual analogue scale

allergenic foods involved and, if needed, supplementation with rescue medication. Here the effect on the quality of life of allergic patients is indisputable. Because Bet v 1–related fruit allergies are usually mild, some patients decide to continue eating apples and related fruits and accept the inconvenience but also take the risk of increasing their allergy. Nevertheless, the majority of patients will avoid offending foods, depriving themselves of important sources of vitamins, minerals, and fibers.

Both Mal d 1 and Mal d 3 are members of the family of the pathogenesis-related proteins.¹⁷ Pathogenesis-related proteins can be induced on pathogen exposure or physical stress. There are several occasions along the path from apple tree to consumption when various kinds of stress can occur related to culture type (organic vs conventional cultivation with differences in pathogen attack), to the physical action of picking the fruit, and to transport and storage conditions. All these factors might potentially influence the level of these pathogenesis-related proteins and, concomitantly, the degree of allergenicity. In addition, natural differences in allergenicity between apple cultivars have been reported. These observations were largely based on verbal patient reports, on *in vitro* methods (eg, ELISA and immunoblotting), and on *in vivo* methods (eg, open oral challenges with pieces of apple and skin prick tests [SPTs] with apple extracts).^{18–20} However, apple extracts used for SPTs have frequently been shown to have poor sensitivity because of the lability of Mal d 1 on extraction.^{21,22}

The aim of this study was to evaluate the usefulness of prick-to-prick SPTs with fresh apples and the double-blind, placebo-controlled food challenge (DBPCFC) in assessing differences in allergenicity between apple cultivars. Identification of low-allergenic cultivars might in the future allow breeders to develop hypoallergenic apples. In addition, the influence of storage conditions was investigated. Elucidation of factors that influence allergenicity during handling and storage might help in the development of improved production protocols that also can be of benefit to patients.

METHODS

Patients

Fifteen adult patients were recruited from the outpatient clinic of the Department of Dermatology/Allergology of the University Medical Center Utrecht (Table I). They all had birch pollinosis manifesting with rhinoconjunctivitis during the birch pollen season (April and May), as well as a positive SPT response to fresh apple (Golden Delicious) of at least half of the diameter of that elicited by the positive histamine control. All patients had a typical history of

TABLE I. RAST results, test participation, and country of origin

Patient no.	Intracultivar and intra-assay variation	DBPCFC	Country	nMal			
				d 1	d 2	d 3	d 4
1	Yes		NL	0.4	0.2	0.1	0.3
2	Yes		NL	0.6	0.2	0.1	0.2
3	Yes		NL	0.6	1.0	0.3	2.8
4	Yes		NL	1.7	0.6	0.7	1.1
5	Yes	Yes	NL	0.6	0.1	0.1	0.3
6	Yes	Yes	NL	0.7	0.2	0.1	0.2
7		Yes	NL	0.6	0.1	0.1	0.2
8			NL	1.0	0.1	0.1	0.6
9		Yes	NL	2.0	0.1	0.1	0.2
10			NL	1.1	0.1	0.1	0.2
11			NL	2.1	0.1	0.1	0.3
12			NL	1.0	0.1	0.1	0.2
13		Yes	NL	1.0	0.1	0.1	0.2
14			NL	5.2	0.2	0.2	0.2
15			NL	2.2	0.1	0.1	0.2
16	Yes		ES	0.1	0.1	1.0	0.3
17	Yes		ES	0.1	0.3	5.6	7.4
18	Yes		ES	0.1	0.2	1.7	0.2
19	Yes		ES	0.1	0.1	1.4	0.2
20	Yes		ES	1.1	0.1	0.1	4.4
21	Yes		ES	0.3	0.3	0.7	0.2
22	Yes		ES	0.7	0.3	3.5	0.7
24	Yes		ES	0.5	0.5	8.9	0.7

Values are presented as international units per milliliter for Mal d 1 through Mal d 4. *NL*, Dutch patients; *ES*, Spanish patients.

apple allergy, with symptoms like itching and mild swelling of the mouth and throat and sometimes rhinoconjunctivitis after eating an apple (oral allergy syndrome). In 5 of the 15 patients, apple allergy was confirmed by means of DBPCFC. In all patients we tested RAST Mal d 1 through Mal d 4 (in international units per milliliter), and RASTs were performed as described previously.^{23,24} For assessment of intra-assay and intracultivar variation, a second group of patients (*n* = 8) was included (Table I). They were randomly selected at Fundación Hospital Alcorcón (Madrid, Spain) from a group of 40 patients with non-birch pollen-related apple allergy confirmed by means of SPT and DBPCFC reactions against Golden Delicious.

This study was reviewed and approved by the Ethics Committees of the University Medical Center Utrecht and of the Fundación Hospital Alcorcón. All patients provided written informed consent before enrollment in the study.

SPT

Fifteen Dutch and 8 Spanish patients with apple allergy were evaluated by means of SPTs, which were performed on the flexor surface of the forearm by using the prick-to-prick-technique according to the method of Dreborg.^{3,25} Histamine dihydrochloride (10 mg/mL) was used as a positive control, and the glycerol diluent of the SPT extracts was used as a negative control (ALK-ABELLÓ, Nieuwegein, The Netherlands). The wheal reaction was measured after 15 minutes and transferred with transparent adhesive tape onto a record sheet. The skin wheal areas were determined by using computer scanning.²⁶

SPT responses were standardized by dividing the original wheal area of a prick by that obtained for the histamine control. All patients

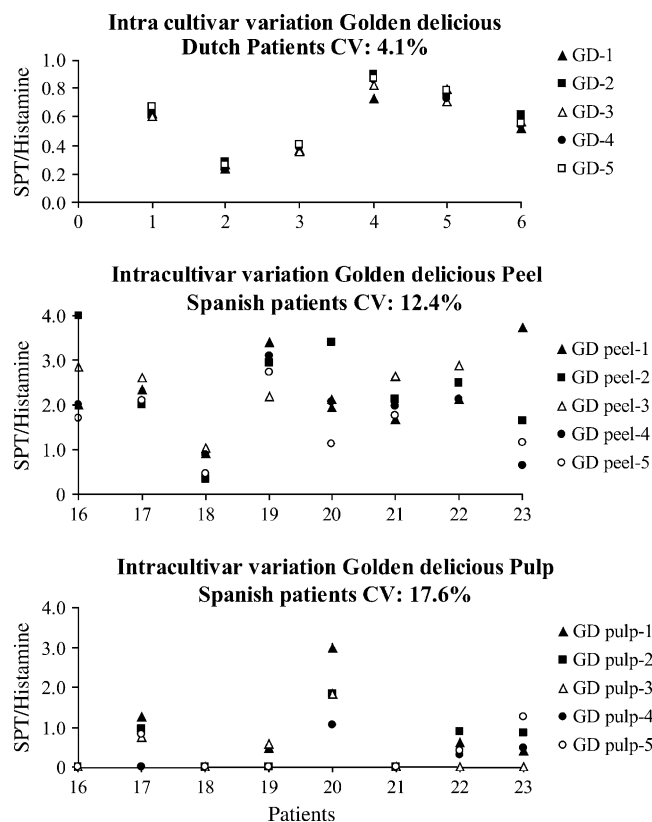


FIG 1. Results are shown for 6 Dutch and 8 Spanish patients with apple allergy using 5 apples of 3 cultivars. Intracultivar variation: Dutch patient, coefficient of variation (CV) of 4.1%. Spanish patients: peel, 12.4%; pulp, 17.6%.

were tested with apples from a single cultivar batch. A single prick per apple was performed for each patient, unless stated otherwise. Golden Delicious was always included as the reference for high allergenicity.

DBPCFCs

Clinical reactivity to the apple cultivars Golden Delicious and Santana was investigated by means of DBPCFC in 5 Dutch patients with birch pollen allergy and allergy to apple. DBPCFCs were carried out as described elsewhere.²⁷ In short, on the day of the challenge, only a light breakfast was allowed no later than 2 hours before the first test meal. The challenges were performed in a clinical research setting equipped for resuscitation and monitoring of vital signs. During the challenge, the investigator or a nurse continuously accompanied the patient. The skin and oral cavity were inspected before the challenge for preexisting lesions and during the challenge when symptoms like itching or (a feeling of) swelling occurred. Between each test meal, an interval was allowed of 15 minutes or longer if needed for (subjective) symptoms to disappear. Patients were kept under observation for at least 1 hour after resolution of symptoms. The severity of the oral allergy symptoms was scored by using visual analogue scales (VASs) with a range of 0 to 100, where 0 is equal to no symptoms and 100 represents severe symptoms.

Challenge meals were prepared within 5 minutes before administration to prevent loss of allergenicity on disruption of apple tissue. They contained 5, 40, and 120 g of fresh shredded apple of each cultivar, respectively. The meals were completed with a mixture of yogurt, orange juice, apple juice, applesauce, and oatmeal flakes. The

placebo doses consisted of the same ingredients, with the exception of fresh apple. Apple juice and applesauce do not contain any IgE-reactive Mal d 1 allergen (Bet v 1 homolog) because of processing and were added for optimal blinding of taste. In addition, patients wore nose clips to mask odor and taste.

Apple cultivars

Nineteen different commercially available apple cultivars, as well as 2 experimental breeding selections, were examined. All cultivars were grown at the research orchard of Plant Research International (Wageningen, The Netherlands). All fruits were harvested at their usual degree of ripeness for consumption. Part of the harvested fruit batch was stored in a single cold room at 2°C under normal atmospheric conditions. In addition, 6 of the cultivars were also stored at 3°C under controlled oxygen (2.5%) and carbon dioxide (1%) conditions.

Statistical analysis

Statistical analyses were carried out on log-transformed SPT values. The log transformation was chosen to obtain observations with an approximate constant variance; the variance of the SPT values is approximately proportional to the mean. For the log-transformed SPT, a value of zero corresponds with the reaction to histamine. Values of less than zero indicate a less severe reaction compared with the reaction to histamine. In all cases, ANOVA was carried out with the computer package GenStat (VSN International Ltd, Herts, United Kingdom). All paired comparisons were made with a 2-sided *t* test.

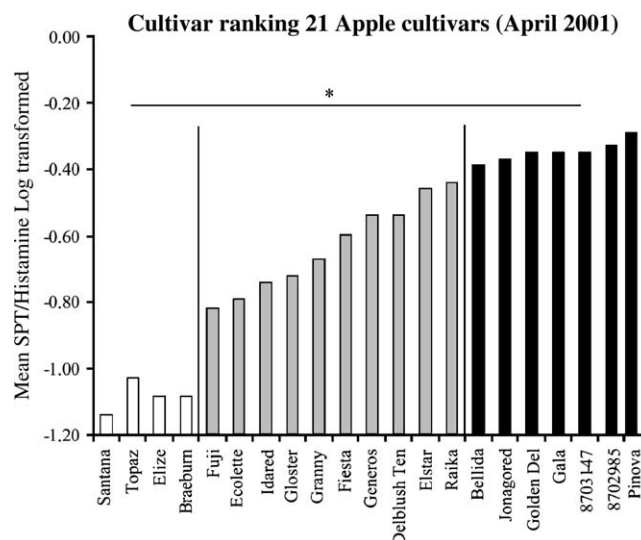


FIG 2. Ranking of 21 cultivars of 9 Dutch patients with apple allergy tested in April 2001. Three categories are shown: low-allergenic (open bars), moderate-allergenic (shaded bars), and high-allergenic (filled bars) cultivars. The low-allergenic cultivars are significantly ($P < .001$) different (*) from the high-allergenic cultivars.

RESULTS

Intra-assay and intracultivar variation

Six of 15 Dutch patients with established birch pollen-related apple allergy and 8 Spanish patients with non-birch pollen-related apple allergy were included in the study to assess the reproducibility of the prick-to-prick method for the determination of allergenicity of apples. All patients were tested with 5 individual apples from each of the following 3 cultivars: Golden Delicious, Fuji, and Ecolette. With each apple, prick-to-prick tests per patient were carried out in duplicate. In case of the Spanish patients, peel and pulp were tested separately for each apple. The intra-assay coefficient of variation among the Dutch patients was 3.9%, and among the Spanish patients, intra-assay coefficients of variation were 11.5% for peel and 14.5% for pulp. Intracultivar coefficients of variation were similar to the intra-assay coefficient of variation (ie, 4.1% for Dutch patients [Fig 1, A, GD] and 12.4% for the Spanish patients when the peel was tested [Fig 1, B, GD]). This shows that differences in SPT responses from apples of the same cultivar batch are very small. For the Spanish patients, the test based on the pulp resulted, however, in a higher coefficient variation of 17.6% (Fig 1, C, GD). Data for Fuji and Ecolette apples are not shown.

In the Dutch patients skin reactivity to Golden Delicious apples was significantly higher than to Ecolette and Fuji apples. For the Spanish patients, the peel of Fuji apples was more allergenic than that of Golden Delicious apples. The peel of Ecolette apples was the least allergenic. These differences were all significant ($P < .05$).

Cultivar ranking

Twenty-one cultivars (Fig 2) were selected for a broader assessment of allergenicity. These were tested on a single day by using the prick-to-prick method in 9 Dutch allergic patients. Fig 2 shows a mean ranking

of the cultivars according to the log-transformed SPT reaction values (ie, the histamine-corrected wheal area). Significant differences ($P < .05$) were found between the responses caused by apples of the high-allergenic ($n = 7$ cultivars) and low-allergenic ($n = 4$ cultivars) categories. The remaining 10 cultivars were classified as of intermediate (moderate) allergenicity, but the difference with high and low allergenicity did not reach significance.

Individual per-patient rankings of allergenicity were determined. Golden Delicious cultivar was ranked in the high tertile for 5 patients and in the middle tertile for 4 patients. Also, Gala cultivar demonstrated an overall high allergenicity ranking: 5 in the high, 2 in the middle, and 2 in the low tertiles. Santana apples were classified as a cultivar with low allergenicity, with 7 rankings in the low, 1 in the middle, and 1 in the high tertiles. Golden Delicious, Gala, and Santana were retested 1 year later to assess the reproducibility of this ranking. Again, these apples had been stored for half a year at 2°C. Prick-to-prick testing was performed in 6 patients, 5 of whom had participated the year before. This testing resulted in a similar ranking: Golden Delicious apples showed the highest mean ranking, followed by Gala apples. Santana apples again demonstrated the lowest allergenicity (Fig 3).

Effect of storage under controlled atmosphere on allergenicity

Five cultivars (Golden Delicious, Gala, Santana, Braeburn, and Fuji) were stored for 6 months either at 2°C under normal air conditions (cold storage) or at 3°C under controlled oxygen (2.5%) and carbon dioxide (1%) conditions (controlled atmosphere storage). In the case of controlled atmosphere storage, the cultivars showed an overall reduction of allergenicity by 15% ($P < .001$) compared with the cold-stored fruits (Fig 4). The order of the cultivars with

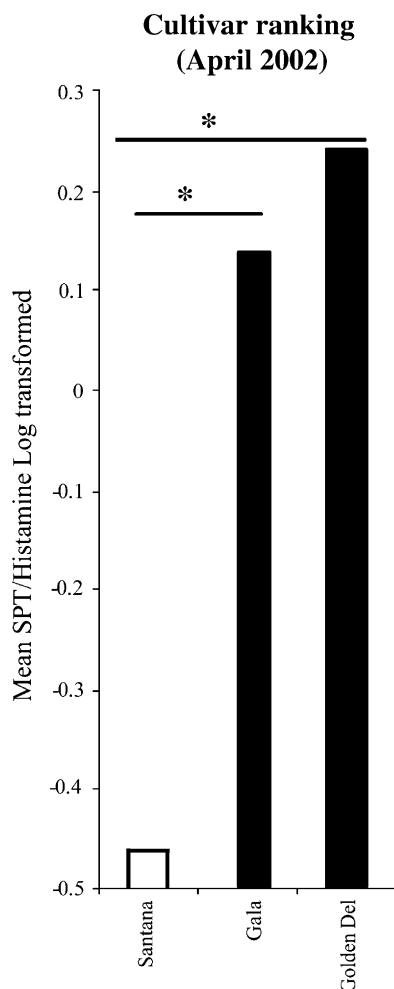


FIG 3. Ranking of 3 cultivars of 4 Dutch patients with apple allergy tested in April 2002. *Open bar*, Low-allergenic cultivar; *filled bars*, high-allergenic cultivars. There is a significant difference (*) between the high-allergenic and low-allergenic cultivars.

respect to the histamine-corrected SPT responses did not change significantly after storage under either condition.

Confirmation by means of DBPCFC of differences in allergenicity

Golden Delicious and Santana apples were used in a DBPCFC experiment in which 5 patients were involved to confirm the observed differences in allergenicity with the prick-to-prick method. All these patients had shown SPT responses for Santana apples to be significantly lower than for Golden Delicious apples ($P = .023$). Fig 5 shows the differences in VAS scores for Golden Delicious and Santana apples after challenging the patients with 4, 40, or 120 g of fruit, respectively. The quantities needed to provoke a similar VAS score were on average 30 times higher ($P < .001$) for Santana than for Golden Delicious apples (Fig 5). These results confirmed that Santana apples were indeed less allergenic than Golden Delicious apples.

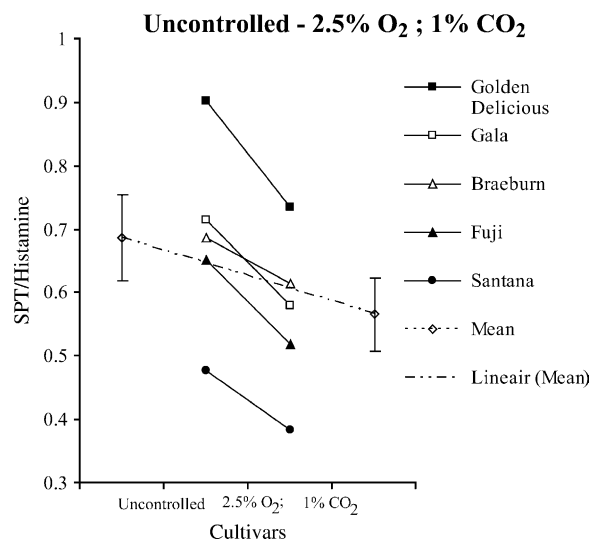


FIG 4. Effect of storage conditions: comparison of cold storage under normal conditions with controlled atmosphere conditions (2.5% oxygen and 1% carbon dioxide). There is 15% ($P < .001$) lower allergenicity under controlled storage conditions.

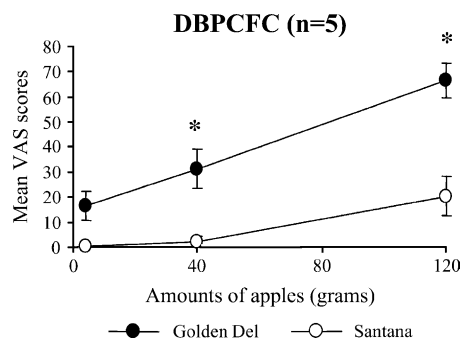


FIG 5. The ranking of allergenicity was confirmed by means of DBPCFC for the cultivars with the highest (Golden Delicious) and lowest (Santana) allergenicity. Results are shown as mean values $\pm$ SEM for 5 patients: *open circles*, Golden Delicious; *filled circles*, Santana. $*P < .05$.

DISCUSSION

The allergenicity of different apple cultivars has thus far mainly been evaluated by means of *in vitro* techniques.¹⁸⁻²⁰ In some cases, extracts from various apple cultivars were used for assessment by means of SPT. In one study clinical evaluation was done by using an open oral challenge.¹⁸ Although precautions were taken to avoid breakdown of labile allergens like Mal d 1, it appears to be difficult to guarantee complete preservation of allergenicity in apple extracts. Some decades ago, Björkstén et al²¹ described a method of preventing loss of IgE-binding potency during extraction of apples. Adding polyvinylpolypyrrolidone and Na-diethyldithiocarbamate inhibited activity of polyphenoloxidases, peroxidases, and proteases, enzymes that are considered to break down the Mal d 1 allergen. This method is still widely used for

the extraction of allergens from plant foods, in particular from fruits. Others have used a low-temperature acetone method with and without addition of enzyme inhibitors.^{22,28} Although these methods make major improvements to the quality of the apple extracts for allergenicity diagnostics, not all the components used (eg, the enzyme inhibitors) are compatible with *in vivo* use. In addition, Mal d 1 reactivity might still be affected. In a recent *in vitro* comparison, the quantity-activity of Mal d 1 was assessed in extracts obtained from more than 75 different European apple cultivars. This experiment was carried out at 2 different laboratories. The results suggested that extracts from one laboratory had lost a significant part of their Mal d 1 activity, despite the fact that both laboratories used an identical extraction protocol with polyvinylpyrrolidone, Na-diethyldithiocarbamate, and a cocktail of inhibitors covering a broad spectrum of proteases (L. Zuidmeer et al, unpublished observations).²⁹ These results indicate that small (as-yet unidentified) differences in extraction conditions can have a very significant effect. To avoid these problems, we decided to use the prick-to-prick method to assess and compare the allergenicity of individual apple cultivars. Of course, there were serious concerns as to whether this *in vivo* test would be reproducible. Five types of variability were expected to exist: (1) the pricking position and the depth of the needle into the apple and subsequently into the patient's under-arm skin³⁰; (2) the amount of apple allergen taken up by the needle and transferred into the skin; (3) differences between individual apples fruits from the same cultivar batch; (4) variation in skin reactivity over the individual patient's arm; and (5) variation of skin reactivity among individual patients. For interpatient comparability, all skin reactions were expressed relative to the reactions observed with histamine. Remarkably, in the Dutch patients with apple allergy, both intra-assay variability and intracultivar variability appeared to be less than 5%. This variability was low compared with the intra-assay variabilities reported for *in vitro* tests like ELISA. However, when tested in Spanish patients, both intra-assay and intracultivar variability were much higher and reached values of around 12% and 18% for peel and pulp, respectively. The most likely explanation for the difference between Dutch and Spanish patients in this analysis relates to the major allergens that are involved, being Mal d 1 in the Dutch patients and Mal d 3 (LTP) in the Spanish patients. It has been reported that LTP is mainly expressed in the peel of apples, whereas Mal d 1 is more homogeneously expressed throughout the fruit.¹⁵ It is therefore to be expected that in the case of Mal d 3, small variations in the depth of pricking into the skin of an apple might largely influence the quantity of Mal d 3 applied to the skin. For Mal d 1, this will have little or no effect. Also, the different ranking for the allergenicity of apple cultivars obtained from the SPT results in the Dutch and Spanish patients can be explained by the different allergens recognized by these different patient groups. This illustrates that allergenicity assessments and rankings for patients with apple allergy from central and northern Europe have little or no

relevance for patients from Mediterranean countries like Spain.

In our study we also performed prick-to-prick tests with freshly harvested apples in October-November (results not shown). From these fruits, generally low allergenic reactions were observed in SPTs, whereas a clear increase was observed for most cultivars after storage for half a year. The occurrence of this difference can be explained as follows. First, SPTs after half a year of storage take place in April-May at the peak of birch pollen exposure. It is well known that specific IgE antibody titers in patients increase during the birch pollen season,^{31,32} which might partly explain the increase in skin reactivity observed for stored apples. Therefore in further experiments, the Mal d 1-specific IgE titers should be determined in the individual patients at the time of the SPT experiment to explain the increased allergenicity as a result of prolonged storage. Second, we were able to establish that prolonged cold storage under controlled atmosphere conditions (2.5% oxygen and 1% carbon dioxide at 3°C) resulted in a significant 15% lower allergenicity than when the apples were stored at 2°C in normal air. On this basis, it would make sense to further investigate various transport and storage conditions, aiming at allergenicity management in apples.

The ranking of 21 apple cultivars presented in this study should be interpreted as a first but reproducible indication. Some of the observed differences did not reach significance because the numbers of patients tested were too small. Nevertheless, the applied prick-to-prick SPT approach combined with the DBPCFC was shown to be a reliable tool for assessment of allergenicity of individual apple cultivars. The observed differences in allergenicity allow differentiation of apple cultivars into high- and low-allergenic categories. Although in the DBPCFC a real threshold dose was not determined, the average 30-fold increase in the dose needed for similar VAS scores suggests that patients with apple allergy can eat approximately 30 times more Santana than Golden Delicious apples before they experience symptoms. This knowledge might offer patients with birch pollen-apple allergy (ie, Mal d 1-reactive patients) a safe way to reintroduce apple into their diets. In addition, the identification of apples with low allergenicity in combination with increased knowledge on the genetic map for Mal d 1 expression and the extent of allelic variation (Gao et al, unpublished data) might allow breeding of novel cultivars with decreased allergenicity. Furthermore, the prick-to-prick SPT approach has also proved to be useful in the selection of *in vitro* grown apple plantlets with reduced allergenicity introduced through Mal d 1 gene silencing by RNA interference.³³

In summary, this study has provided support for the usefulness of the prick-to-prick SPT and the DBPCFC in the evaluation of the allergenicity of fruits and demonstrated the existence of significant differences in allergenicity between apple cultivars, including storage effects. These findings will help to develop improved food allergy prevention strategies.

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