

Measurement of Lipid Transfer Protein in 88 Apple Cultivars

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Key Words

Allergen quantification · Allergenicity · Apple · Basophil histamine release · Cultivar screening · Double-blind, placebo-controlled food challenge · Lipid transfer protein · Mal d 3 · Skin prick test

Abstract

Background: Fruits are a major cause of food allergy in adults. Lipid transfer proteins (LTP) are implicated in severe allergic reactions to fruits, but little is known about LTP content in different cultivars. **Objective:** Determination of the levels of LTP in a wide range of apple cultivars. **Methods:** LTP was measured in apples from 53 cultivars grown in Italy and 35 grown in The Netherlands, using three different immunoassays: a competitive ELISA (cELISA), a sandwich ELISA (sELISA) and a RAST inhibition (RI). Selected cultivars were evaluated using the basophil histamine release test (BHR), skin prick test (SPT) and double-blind, placebo-controlled food challenge (DBPCFC). **Results:** LTP levels measured with the three immunoassays were significantly correlated, as judged by Pearson's correlation ($0.61 < R_p < 0.65$; $p < 0.0001$), but differed with respect to the actual quantities: 3.4–253.2 (sELISA), 2.7–120.2 (cELISA) and 0.4–47.3 $\mu\text{g/g}$ tissue (RI). Between cultivars, LTP titers varied over about a two-log range. Pilot in vitro and in vivo biological testing (BHR, SPT and DBPCFC) with selected cultivars supported the observed differences in LTP levels. **Conclusions:** Around 100-fold differences in LTP levels exist between apple cultivars. Whether the lowest observed levels of LTP warrant designation as hypo-allergenic requires more extensive confirmation by oral challenges. Determination of cultivar variation in LTP levels provides important information for growers and consumers. Comparison to earlier reported Mal d 1 levels in the same cultivars reveals that a designation as low allergenic does not always coincide for both allergens.

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Introduction

Apples are widely consumed and promoted as part of a healthy diet. More than 7,500 apple varieties exist worldwide (http://en.wikipedia.org/wiki/List_of_apple_cultivars). Unfortunately, they pose a serious allergenic risk to some individuals [1]. Allergy to fruits ranks among the most common food allergies in adults [2]. In Central and Northern Europe, allergy to apple (and other fruits and nuts) is mostly associated with birch pollen allergy and is almost exclusively mild and restricted to the oral cavity [3, 4]. Primary sensitization to the major birch pollen allergen Bet v 1 results in cross-reactivity to its homologue in apple, Mal d 1 [5, 6]. In Southern Europe, sensitization to lipid transfer protein (LTP) plays a dominant role in apple allergy. Sensitization to apple LTP (Mal d 3) is a very significant risk factor for severe apple allergy [7–11].

At present the only treatment for food allergy is avoidance and rescue medication. Avoidance of fruits like apples is especially relevant for those patients that are sensitized to LTP. Another approach may be the identification of low-allergenic apples, especially since differences in allergenicity have been described for apple cultivars. Most of these studies focused on Mal d 1, and consequently designation of cultivars as hypo-allergenic only has relevance for patients with birch pollen-related apple allergy. Apples with a low Mal d 1 content can potentially decrease the need of strict avoidance in such groups [12]. Moreover, knowledge about genotypic differences in Mal d 1 levels in different cultivars can support strategies to breed novel low-allergenic cultivars. A recent study reported Mal d 1 levels in 88 different apple cultivars, but at the same time demonstrated discrepancies between results obtained with different assays for the measurement of the allergen [13]. These discrepancies were largely explained by differences in assay fine specificity, and to some extent by interlaboratory variation. That study clearly illustrated how complex the interpretation of allergen measurements can be, in particular for a labile allergen like Mal d 1.

Limited information is available about LTP levels in apple cultivars. The link between LTP and severe allergies makes such information highly relevant, especially since the apple cultivar Santana, containing low amounts of Mal d 1, has recently been marketed in The Netherlands (where the majority of the apple-allergic population suffers from Mal d 1 allergy) as 'suited for persons with mild apple allergy'. Such cultivars may not have increased safety for individuals with LTP allergies. In a recent study

we showed differences in LTP levels among a small panel of apple cultivars [14]. In a Spanish study by Carnes et al. [15], the allergenic profile of 10 apple cultivars was compared on the basis of in vitro IgE reactivity and skin prick tests (SPT). Furthermore since LTP is a pathogenesis-related protein its expression may be affected by environmental factors although this assumption needs confirmation in future studies.

We were interested to know whether apple cultivars exist that are low allergenic to LTP-allergic subjects. As it is not feasible to test high numbers of cultivars by provocation tests, we have first undertaken a comprehensive study to determine LTP levels in the 88 apple cultivars for which Mal d 1 levels were previously reported, again using three different immunoassays for allergen measurement, to increase the validity of observed differences. Confirmation of potential hypo-allergenicity will need to come from future in vivo studies using double-blind, placebo-controlled food challenges (DBPCFC). In the present study, DBPCFC and SPT, as well as an in vitro basophil histamine release test (BHR), were piloted on a few selected cultivars.

Materials and Methods

Sera

For RAST inhibition (RI), a mixture of two Dutch in-house reference sera (PF194 and 227) with IgE antibodies specifically directed to apple LTP (5.7 and 9.3 IU/ml, respectively) were used [16, 17]. All patient sera used for the BHR (n = 2), SPT (n = 13) and DBPCFC (n = 4) had a convincing history and positive SPT for apple (Golden Delicious) and IgE antibodies against apple LTP. The patients used for SPT and DBPCFC were randomly selected from a group of patients with non-pollen-related apple allergy (positive history and CAP to Pru p 3/Mal d 3 and negative CAP to Bet v 1/Bet v 2, no specific IgE to Mal d 1 or Mal d 4). The study was performed with the approval of the local ethics committees, and with consent of the patients.

Apple Extracts

Apples of 35 different cultivars from Wageningen (The Netherlands) and 53 cultivars from Ferrara (Italy) were harvested, stored and extracted as described previously [13], essentially according to the method of Bjorksten [18]. Five-millimeter-thick peels were used from 5 apples per cultivar.

Immunodetection of Apple LTP

A *competitive ELISA (cELISA)* was performed as described earlier [14]. Purified natural apple LTP [19] was coated onto microtiter plates. Binding of apple LTP-specific rabbit IgG [20] was inhibited by apple extracts. Purified apple LTP was used as a standard.

A *sandwich ELISA (sELISA)* was performed as described previously [20]. Apple LTP-specific mouse monoclonal antibody 6B5

was used as capturing antibody and rabbit polyclonal antiserum against apple LTP as source of detecting antibodies.

RAST inhibition assay was based on the RAST procedure described previously [21]. Purified natural apple LTP (100 µg) [19] was coupled to 100 mg of CNBr-activated Sepharose 4B (Amersham Biosciences, Uppsala, Sweden). A mixture of sera (PF194 and 227) with IgE antibodies directed to apple LTP (as confirmed by immunoblot, results not shown) was pre-incubated for 2 h with serial dilutions (in 50 µl PBS/0.3% BSA/0.1% Tween-20) of apple extract prior to the addition of Sepharose-coupled apple LTP. Subsequent steps were identical to those described for RAST [21]. For all three assays, calculation of LTP contents was performed by parallel-line analysis using a standard curve of natural apple LTP. LTP concentrations were expressed as micrograms LTP per gram of peel (wet weight).

Basophil Histamine Release Assay

White blood cells were isolated from the blood of a non-allergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere [22, 23]. Subsequently cells were resensitized with patient's serum. Histamine release was performed with apple extracts from three selected cultivars (Priscilla, Granny Smith and Topaz). Liberated histamine was measured by the fluorometric method essentially as described by Siraganian [24]. Stripped cells were used as a negative control and induced a histamine release lower than 3% (data not shown). The protocol was approved by the medical ethical committee of the Amsterdam Medical Center under project number: MEC97/030.

Skin Prick Test

Spanish patients with apple allergy volunteered for evaluation of apple cultivars by SPT. SPT was performed as described previously [12] with the prick-to-prick method according to Dreborg and Frew [25]. The following cultivars were tested (the number of patients tested are given in parentheses); Ecolette (13), Golden Delicious and Fuji (8), and Santana and Topaz (5). Peel and pulp were tested separately (in duplicate). The results presented correspond to the apple peel data, as the peels contain the most LTP [26, 27]. Histamine dihydrochloride (10 mg/ml) was used as positive control, and saline as negative control (ALK-Abello, Madrid, Spain). The wheal reaction was measured after 15 min and transferred with transparent adhesive tape onto a record sheet. The skin wheal areas were determined using computer scanning. Wheals were expressed in square millimeters.

Double-Blind, Placebo-Controlled Food Challenge

DBPCFC were conducted in the hospital setting as described previously [28]. The same recipe was used to blind apple. Active and placebo were randomly administered on separate days. Challenge foods were prepared immediately before the challenge and four doses (5, 25, 75 and ~250 ml) were administered at 30-min intervals. After the last dose, patients remained under observation for 2 h.

Statistical Analysis

Mean values, standard deviations and ranges were calculated for the results obtained in the three assays for the immunodetection of LTP. Mean values of LTP contents obtained by the three assays were compared by Student's *t* test and paired correlations between them were analyzed using Pearson's *r* test. Differences in

skin reactivity to the five cultivars were analyzed by factorial analysis, and their adjusted mean values and 95% confidence intervals were calculated. With a *p* value < 0.05, a difference was regarded as significant.

Results

Variation in Apple LTP Content and Differences between Assays

Extracts of 88 apple cultivars grown in Italy or The Netherlands were analyzed for LTP content using three different immunoassays. Measured LTP levels were highest in the sELISA, ranging from 3.4 to 253.2 µg/g tissue, with a mean ($\pm$ SD) of 66.8 ($\pm$ 53) µg/g. On average the titers were three times lower (21.2 $\pm$ 17.7) when measured by cELISA, ranging from 2.7 to 120.2 µg/g, and about six times lower (11.1 $\pm$ 9.8) when evaluated by RI, ranging from 0.3 to 47.3 µg/g. Together, this demonstrates that the contents of LTP observed for the different cultivars span about two orders of magnitude. Differences between the means of the three assays were significant in all cases (*p* < 0.0001). All assays demonstrated significant correlation as judged by Pearson's correlation (0.61 < *R_p* < 0.65), supporting that they all measure the same allergen (fig. 1). The strongest correlation was found for RI and the cELISA (*R_p* = 0.65). Overall LTP levels detected in five cultivars (Golden Delicious, Granny Smith, Braeburn, Fuji and Jonagold) grown in both countries were similar (fig. 2), although in some cases there were clear differences, even up to >10-fold, as observed for Jonagold and Fuji when measured by cELISA (table 1).

In vitro and in vivo Biological Activity of Selected Cultivars

To evaluate whether observed differences in LTP content (table 1) translated into differences in in vitro (BHR) and in vivo (SPT and DBPCFC) biological activity, small pilot studies were performed using seven apple cultivars (Priscilla, Ecolette, Golden Delicious, Fuji, Santana, Granny Smith and Topaz). These cultivars were chosen on the basis of their determined LTP content in the in vitro tests and their availability during the test period. To evaluate whether differences in LTP content were reflected in in vitro biological activity, the extract of a low LTP cultivar (Priscilla) was compared to the extracts of two high LTP cultivars (Granny Smith and Topaz). By sELISA and RI, Priscilla was around 30- and 50-fold reduced in allergenicity compared to Granny Smith and Topaz, respectively. By cELISA these differences were 9- and 14-fold. In BHR, decreased allergenicity was confirmed, al-

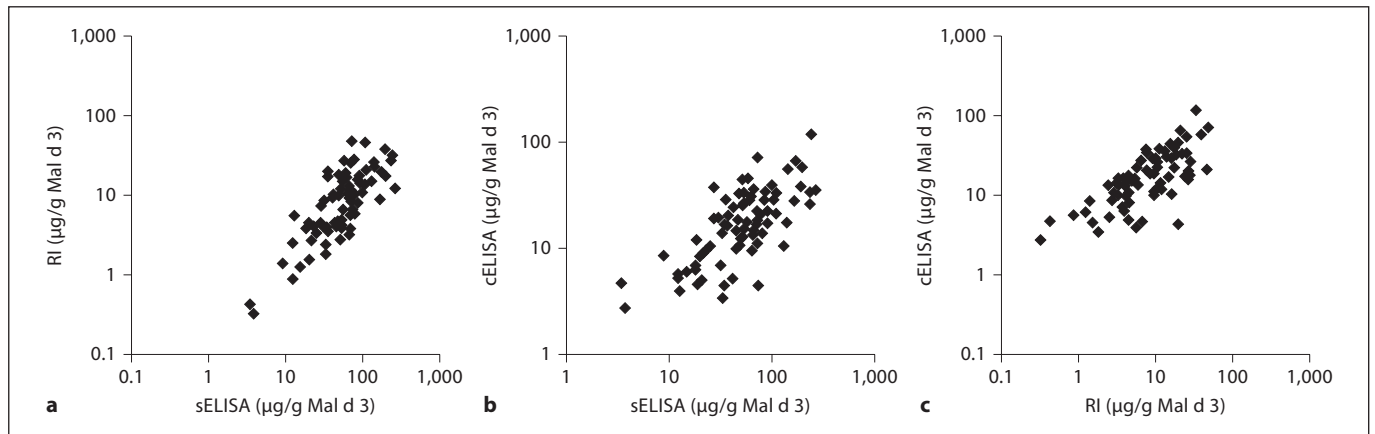
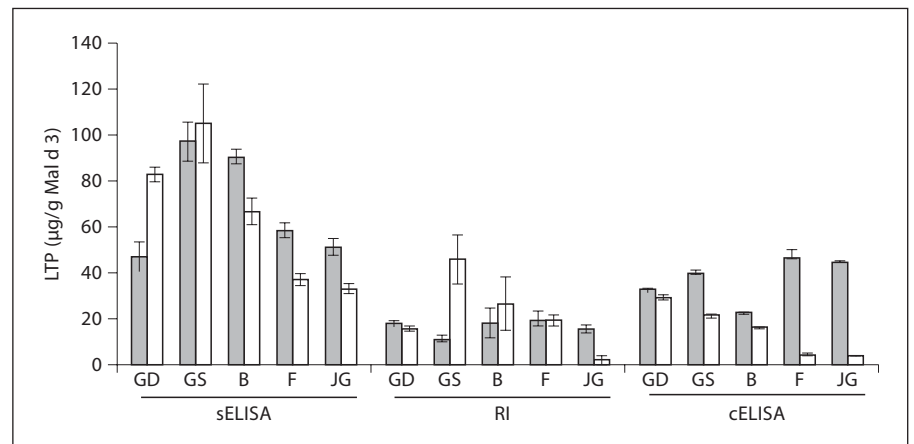


Fig. 1. Correlation between LTP levels ($\mu\text{g/g}$ tissue) detected with the sELISA and the RI (a) and cELISA (b), and RI and the cELISA (c).

Fig. 2. LTP levels ($\mu\text{g/g}$ tissue) in apples of five cultivars grown in The Netherlands (shaded light grey) and Italy as determined in the three assays. GD = Golden Delicious; GS = Granny Smith; B = Braeburn; F = Fuji; JG = Jonagold.



though not to the extent observed by sELISA and RI (fig. 3). For 1 patient, the decrease in biological activity was 4.4- and 6.9-fold, for another 16.3- and 13.0-fold. SPT and DBPCFC were carried out with fresh apples from different harvests as the ones that were used for the extracts analyzed for LTP content. SPT was carried out using five cultivars. In order of increasing SPT reactivity these were Santana, Ecolette, Topaz, Golden Delicious and Fuji (fig. 4). Differences between the three cultivars with higher SPT activity were not significant. Differences in these cultivars with both cultivars (Santana and Ecolette) showing lower SPT reactivity were significant. The designation of Santana and Ecolette as low allergenic corresponds with the three immunoassays with the exception of Santana in the sELISA (table 1). Finally, three of the cultivars evaluated by SPT were selected for a DBPCFC in 4 patients. Unfortunately, patient compliance (evalua-

tion of three cultivars requires patients to come to the hospital on 4 separate days) resulted in an incomplete evaluation (table 2). One patient tolerated Ecolette up to the highest dose but reported oral allergy syndrome (OAS) when challenged with Topaz, and 1 patient tolerated Santana while reporting OAS for Topaz. These observations are in line with the immunoassays and SPT. The latter patient did report OAS to Ecolette. In the end, 2 patients declined the evaluation with Topaz.

Discussion

As has been reported in an earlier survey for Mal d 1 contents of apple cultivars, different assays for the measurement of LTP gave different results. Although the assays correlated quite well, absolute quantities differed, de-

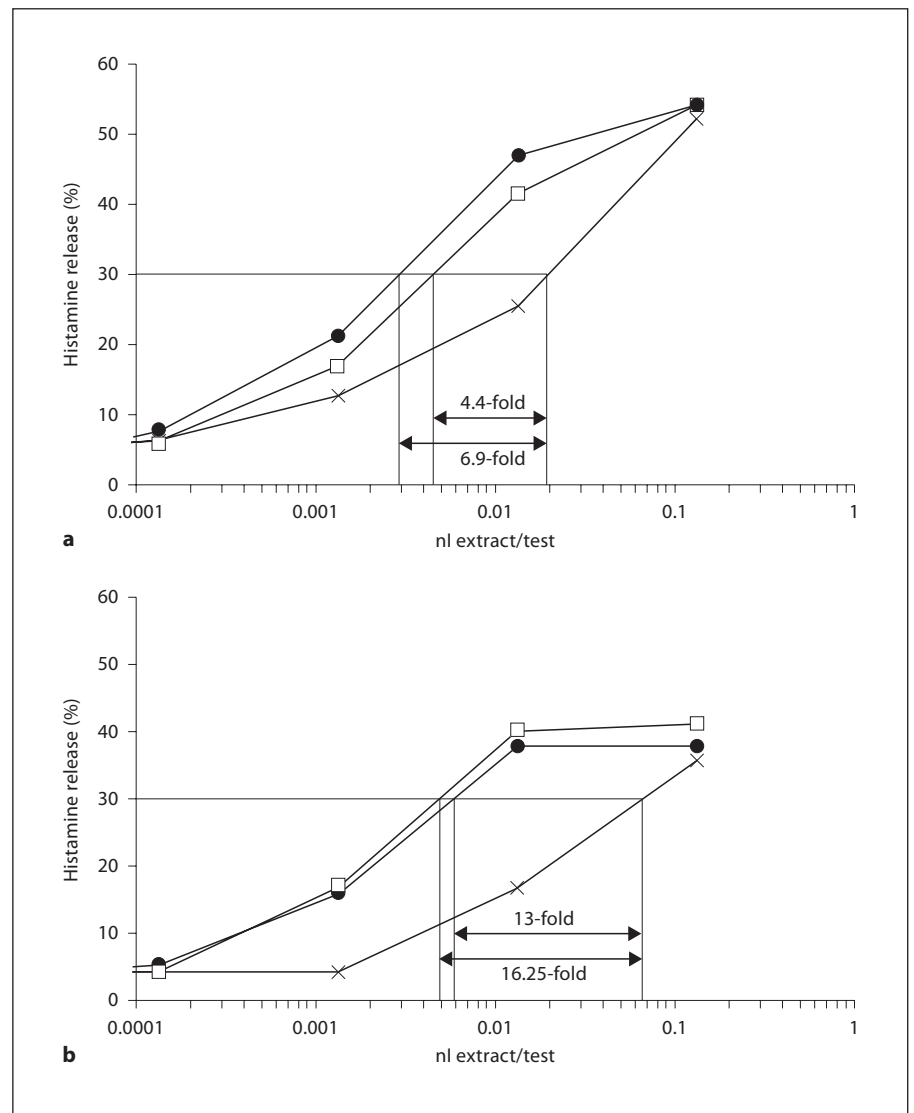


Fig. 3. BHR using apple cultivars Priscilla (x), Topaz (●) and Granny Smith (□) with serum of patients S008 (a) and I80 (b).

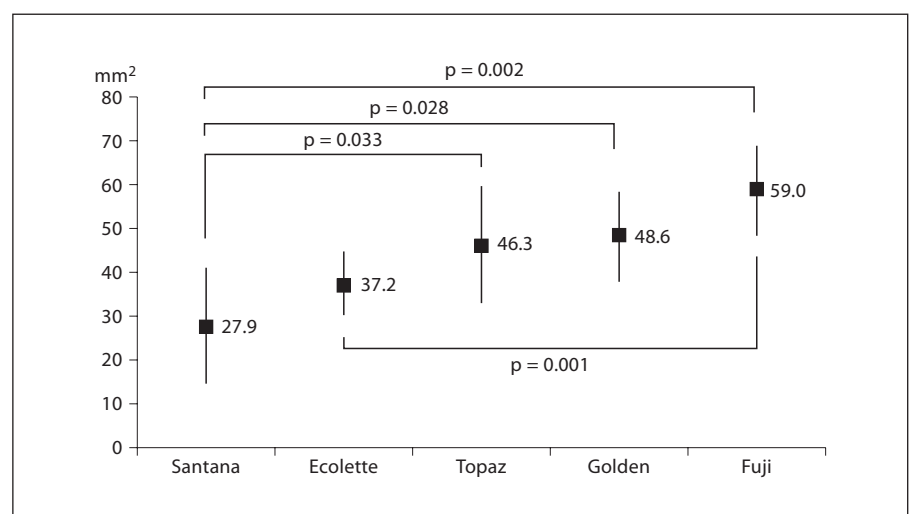


Fig. 4. Ranking of five cultivars (Santana, Ecolette, Topaz, Golden Delicious and Fuji) based on SPT data. The histamine-adjusted mean levels are indicated; bars indicate the upper and lower limits of the 95% confidence interval. Significant p values (< 0.05) are indicated.

Table 1. LTP levels ($\mu\text{g/g}$ tissue) in selected cultivars

Cultivar	Biological test	The Netherlands			Italy		
		sELISA	cELISA	RI	sELISA	cELISA	RI
Priscilla	BHR	3.4	4.6	0.4			
Ecolette	SPT, DBPCFC	30.2	18.7	8.6			
Golden Delicious	SPT	46.9	32.6	18.0	82.7	29.2	15.7
Jonagold	–	50.9	44.8	15.3	32.7	3.4	1.8
Fuji	SPT	57.8	46.6	18.9	37.0	4.4	19.0
Santana	SPT, DBPCFC	70.3	16.3	5.4			
Braeburn	–	90.3	22.3	17.6	66.6	15.8	26.3
Granny Smith	BHR	97.0	39.9	11.0	104.8	21.3	45.9
Topaz	SPT, DBPCFC, BHR	164.7	65.6	20.7			

The table lists cultivars that were selected for evaluation of biological activity and a partly overlapping selection of cultivars that were grown in both countries. The cultivars are listed in order of increasing LTP content measured by sELISA. Higher LTP values are indicated by increased darkness of grey shading.

spite using the same purified protein for calibration in the three assays. The sELISA gave approximately three times higher results than the cELISA and six times higher than the RI. The monoclonal antibody used as capturing agent in the sELISA has been reported to be cross-reactive to other fruit LTP, i.e. to recognize a highly conserved epitope on the LTP molecule [20]. It is therefore likely that different isoforms [29] of LTP are captured with similar sensitivity and subsequently detected by polyclonal rabbit antibodies. In both competitive assays (cELISA and RI), some isoforms are possibly underrepresented in the immobilized purified LTP, resulting in decreased sensitivity for detection. Additionally, the even lower allergen concentrations observed in RI compared to the cELISA suggest that IgE epitopes might be relatively labile in extracts compared to the purified standard. For Mal d 1, poor conservation of IgE-binding epitopes in extracts is well established [13, 30]. The results described in this study suggest that this may – to a lesser extent – also be true for LTP. Despite such discrepancies with regard to absolute quantitation, the results of the current study provide a good indication of the order of magnitude of LTP levels in different cultivars; cultivars ‘low’ and ‘high’ in LTP concentrations roughly differ up to 100-fold, irrespective of the type of assay used. However, when ranking apple cultivars for allergenicity, we have to keep in mind that many other factors such as time or season of harvesting, length and method of storage (reported to maximally result in a 2-fold difference) and even the position on a tree (another 2-fold difference) can influence allergenicity [14]. Although our findings were not significant, there is an indi-

Table 2. DBPCFC with selected apple cultivars

Patient	DBPCFC			
	Ecolette	Santana	Topaz	Placebo
1	neg.	ND	OAS	neg.
2	OAS	neg.	OAS	neg.
3	ND	OAS	ND	neg.
4	OAS	OAS	ND	neg.

ND = not done; neg. = negative.

cation that these differences may also exist within one cultivar grown in two different countries and thus other agronomic differences can play a role in allergenicity. In fact, this ‘biovariability’ poses a major problem for accurate assessment of cultivar allergenicity, as there are so many different factors influencing this allergenicity that it is impossible to give a ‘true’ ranking. However, for patients and apple breeders, it is of great importance to know which apples may be at the lower end of the spectrum of LTP content. Differences observed by measuring LTP are of a different order of magnitude (at least 10- to 100-fold) than those induced by growing conditions, harvest time and storage conditions (<5-fold). This implies that the present data can be used to select candidate cultivars for further in depth in vivo evaluation of potential hypo-allergenicity. A protocol for such studies is currently being developed, and will include cultivars like Priscilla, Santana and Ecolette as hypo-allergenic candidates, and

some high-allergenic cultivars for reference purposes (e.g. Topaz, Granny Smith and Fuji). Preliminary data from the present study have confirmed that BHR, SPT and DBPCFC are sensitive enough to detect differences in allergenicity between different apple cultivars. Using BHR, a clear distinction was made between Priscilla (low allergenic) on the one hand and Granny Smith and Topaz (high allergenic) on the other hand. Also in SPT two low-allergenic cultivars (Ecolette and Santana) were significantly less reactive than three high-allergenic cultivars (Golden Delicious, Fuji and Topaz). From the pilot DBPCFC performed in this study, it is impossible to draw firm conclusions. The major problem is that for a statistically significant study over 50 patients are needed. In addition, patient compliance can pose a problem, as performance of DBPCFC involves 2 separate days for verum and placebo. This means that patients will need to come to the hospital for 4 days for the evaluation of three cultivars. Obviously, this was too much for the volunteers participating in the present study, because only 1 patient completed the 4 challenge days. The other 3 in the end declined one or two challenges. Nevertheless, for both Santana and Ecolette, it could be confirmed in a single patient that these were tolerated in contrast to Topaz. Patients reacted with OAS, which is the most common type of clinical presentation of LTP allergy in two thirds of the Spanish patients in 'real life', despite the fact that LTP sensitization is a risk factor for severe symptoms [7]. This is in line with challenges performed for routine diagnosis at the outpatient clinic, where OAS is reported in ~80% of the patients as the sole manifestation [unpubl. obs.].

Whether LTP levels at the lower end of the spectrum ($<5 \mu\text{g/g}$) are sufficiently low not to trigger an allergic reaction in an LTP-allergic individual remains to be determined by *in vivo* studies. A (first) bite of an apple will be around 10 g of which around 4.5 g is ~5-mm thick peel. This implies that a bite of a low-allergenic apple like Priscilla will expose an allergic patient to around 15 μg of LTP, and of a high-allergenic apple to around 1.1 mg, with consumption of an entire 200-gram apple resulting in someone eating either 300 μg of allergen from a low-allergenic cultivar or 22 mg from a high-allergenic cultivar. Whether this is below the threshold for inducing an allergic reaction can only be established by DBPCFC in a statistically significant number of LTP-allergic patients. Interestingly, analysis of the minimum amount of apple reported to trigger a reaction in Spanish apple-allergic patients (DBPCFC positive to apple; nMal d 3 RAST >0.30 ; $n = 57$) [7] revealed that in 54.4% the reaction appeared after a single bite, 12.3% after having eaten half an

apple, and 33.3% needed to eat the whole apple to have a reaction [Fernández-Rivas, pers. commun.].

The variation in Mal d 1 levels in this same apple cultivar extracts were reported earlier [13]. The maximum difference between low- and high-allergenic cultivars for Mal d 1 was similar to that now reported for LTP: up to 100-fold. As in the present study for Mal d 3, an accurate ranking for Mal d 1 was not made due to the interassay variability. Additionally, in the Mal d 1 study, a significant interlaboratory difference was reported for extracts produced from the Italian and Dutch cultivars, most likely linked to the extreme lability of Mal d 1 compared to LTP. Therefore, we can only compare the 'Mal d 1 ranking' and the 'LTP ranking' from the separate countries individually. If we divide the allergen levels per cultivar (mean values from the three assays) for Mal d 1 and for LTP into quartiles (high, medium/high, medium/low and low Mal d 1 or LTP content), 12/35 Dutch cultivars (34.3%) and 20/53 Italian cultivars (37.7%) fell into the same group for both allergens. Nine of 35 (25.7%) and 19/53 (35.8%) cultivars show a difference of more than one quartile. Thus an apple that has a low Mal d 1 content is not by definition equivalent to one with a low LTP content. Topaz is in the highest quartile for LTP and in the third quartile (medium/low allergenic) for Mal d 1. A similar difference was observed for Braeburn. The cultivar Santana, which was shown to be hypo-allergenic for Mal d 1-reactive patients [12] and is now piloted on the Dutch market as such, was ranked in the third quartile (medium/low) for both Mal d 1 and LTP. In the SPT, Santana was the lowest of five cultivars tested, confirming its ranking as medium/low allergenic also for LTP-sensitized patients. The DBPCFC results are too limited to be able to draw a firm conclusion about the allergenicity of Santana for LTP-allergic patients. Whilst sensitization to apple LTP is rare in The Netherlands [7], a cultivar like Santana could be a solution for Mal d 1-reactive patients with only mild symptoms. Whether Santana or even more likely Priscilla is a hypo-allergenic apple for LTP-allergic patients needs to be established in a sufficiently powered DBPCFC trial. Our results thus support the widespread view that specific advice regarding which foods are safe for an individual to eat needs to be provided by an allergist since the individual allergy of foods have to be taken into account.

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