ORIGINAL ARTICLE

Allergen-specific immunotherapy with apples: selected cultivars could be a promising tool for birch pollen allergy

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Abstract

Background Most birch pollen-allergic patients develop allergic cross-reactions to the major allergen found in apples Mal d1, known as pollen-related food allergy (prFA). This is due to a strong clinically relevant homology between the major allergen in birch Bet v 1 and Mal d 1. Daily apple consumption induces oral tolerance in prFA, but its effect on the inhalational allergy has not been investigated.

Objectives As continuous apple consumption might also mitigate the inhalational allergy, this study aimed to uncover apple cultivars suitable for treatment of birch pollen rhinoconjunctivitis and apple allergy in a controlled and established dosage.

Methods Patients (n = 52) with birch pollen allergy and prFA to apples were subjected to a prick-to-prick test (SPT) with 23 cultivars (red-fleshed, old traditional and new commercial). By SPT, the apple parts flesh, peel equatorial and peel apical near the stalk were compared for their reactivity. One apple cultivar of each allergenicity class (low, middle and high) was subsequently tested in an oral provocation test (OPT).

Results According to the SPTs, we provide a ranking of all 23 cultivars. Red-fleshed apples displayed the lowest reactivity, followed by old and new cultivars. Four cultivars showed disagreement from their allergenicity class: Santana and Pink Lady®, new cultivars that provoked only low to moderate. In contrast, White Rosemary and Goldparmäne, two old cultivars, induced strong reactions. Skin reactivity increased from flesh to peel to stalk, and SPT results could predict the severity of prFA of each allergenicity class.

Conclusions Herein, we propose a treatment protocol for allergen immunotherapy to birch pollen and prFA with daily apple consumption. Red-fleshed, old and the new cultivars Santana and Pink Lady® provoke less allergic reactions and are suitable for initial induction. After a controlled and well-tolerated increase of intake, also moderate and finally high allergenic apple cultivars should be integrated into treatment of birch pollen allergenic patients.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Introduction

Apples are one of the most consumed fruits in Europe, available throughout the year and containing high amounts of polyphenols with positive effects on human health. Unfortunately, most birch pollen-allergic patients also suffer from an allergy to raw apples, other *Rosaceae* fruits, vegetables or nuts, known as oral allergy syndrome (OAS) or birch pollen-related

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food allergy (prFA).[1] The patients display typical symptoms including itching, scratching or swelling of the mouth, throat and lips immediately after eating and preventing them to eat raw apples. This is caused by a strong clinically relevant homology between Bet v 1 and Mal d 1,[2] the major allergens in birch and apple, and provides an opportunity to use the Mal d 1 in apples to potentially cure both birch pollen- and pollen-related apple allergy. Successful sublingual immunotherapy (SLIT) for allergic rhinoconjunctivitis with birch allergen preparations could be shown and,[3,4] however, had only limited effects to prFA.[1,5] On the other hand, oral desensitization to prFA could be directly achieved by apple consumption,[6,7] but it's effects on the inhalational allergy have not been thoroughly investigated. It has already been shown that Bet v1 and Mal d1 have a very similar three-dimensional structure and share reactive IgE and T-cell epitopes.[2,8,9] SLIT with recombinant Mal d 1 downregulates the allergen-specific Th2 response to both Mal d 1 and Bet v 1 and also suppresses Bet v 1-specific, cross-reactive T cells.[10] Further, oral exposure to Mal d 1 reduced Bet v 1 specific serum IgE levels and induced immune responses seen during peripheral tolerance development.[9,11] Anecdotally after hearing from the project, one author (KE) was contacted by 3 previous birch pollen-allergic patients with prFA that had desensitized themselves by continuous daily long term (all over 8 months and ongoing) consumption of a half to one high allergenic apple. So, if feasible allergen immunotherapy (AIT) by eating apples would provide a healthy, time saving and convenient way for allergy treatment, in comparison with an elaborate long-standing conventional specific immunotherapy against birch pollen.

Notwithstanding, all apples are not the same. The apple's domestication led to thousands of cultivars with different properties in colour, size, taste and allergen content. Different apple cultivars differ in their Mal d1 content, but Mal d1 content also varies within the apple increasing from flesh to peel and stem near peel.[12–20] Previous studies associated high Mal d 1 content with an increased allergenic potential.[13,21,22] The protein itself belongs to the pathogenesis-related (PR) 10 family, involved in plant pathogen defence.[17] Therefore, patients with prFA to apple often report tolerating peeled but also local, traditional apple sorts or so-called 'old cultivars' from their garden much better than new, commercial apples that primary emerged after the postgreen revolution in the 1960s where breeders started to develop new improved sorts with increased pathogen resistance.[13]

The AppleCare Study aims to propose a treatment protocol for AIT to birch pollen and prFA by daily apple consumption, after the identification of suitable apple cultivars, dosages and duration of intake. We, therefore, analysed the allergenic potential of red-fleshed, old and new apple cultivars by testing different apple parts onto the skin and subsequently selected and tested suitable therapy cultivars by oral provocations.

Materials and methods

Study design

In 2017, 52 participants (31 Austrian and 21 Italian patients) with birch pollen-related apple allergy aged 18 to 70 years were recruited at the Departments of Dermatology, Venerology and Allergology of the Medical University of Innsbruck (Austria) and the Central Teaching Hospital of Bolzano (Italy). The participants displayed allergic rhinitis in spring, positive prick test to birch pollen extract (ALK-Abelló, Madrid, Spain), specific Bet v 1 and Mal d 1 IgE levels greater than 0.35 kU/L (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) and OAS symptoms to fresh apples. Participants sensitized against the heat-stable lipid transfer Mal d 3 protein (> 0.35 kU/L) with the potential to induce severe anaphylactic reactions or who had received birch pollen AIT within the past 5 years were excluded. The study was performed with the approval of both local ethics committees (Innsbruck and Bolzano/Bozen) and with the written consent of the participants.

Apple cultivars

Twenty-three different apple cultivars were selected to define the allergenicity of red-fleshed, old and new cultivars, listed in Table 1. All the cultivars used in the SPT were provided by Laimburg Research Centre and grown in the region near Bolzano (IT). Five different red-fleshed apple cultivars, named Bay 3484 Baya® Marisa (BM), Y103 Kissabel® (RF2), R201 Kissabel® (RF4), RS-1 Red Moon® (RM) and Luresweet Redlove® (RL), were tested. Old and new apple varieties were distinguished according to Vegro *et al.*[17] New cultivars are defined as those selected and optimized by humankind, particularly 'after the postgreen revolution' in the 1960s, including Bonita (BO), Elstar Lb®87/1 (EL), Fuji Zhen® (FJ), Gala Buckeye® (GA), Gloster (GO), Golden Delicious Klon B (GD), Lb 17906 (LB), CIVG198 Modí® (MO), SQ159 Natyra® (NA), Pink Lady® Rosy Glow (PL), Santana (SA), Bay 4210 Sonnenglanz® (SG) and Topaz (TO). The old apple cultivars include five varieties cultivated and appreciated 'before the green revolution' named Goldparmäne (GP), Kanada Renette (KR), Tiroler Spitzlederer (TS), White Rosemary (WR) and White Winter-Calville (WW).

For the SPT, apples were harvested at physiological ripening stage in South Tyrol (IT) in 2017. Subsequently, cultivars were kept in cold storage at 2°C for 11 days followed by 3 days shelf life at 20°C. The apples were immediately sliced in cubes and shock frozen according to Vegro *et al.*[17] Samples from the flesh and peel (equatorial) were taken from all 23 cultivars. Additionally, peel near the stalk (apical) was taken from BO, FJ, GO, GD, KR, LB, MO, NA, RL, PL, TS, TO, WR and WW. Cubes were stored at −80°C until use. The cultivars EL and GP were tested only in Austria.

Table 1 Apple prick test panel

		Cultivar	Abbr.	Positive prick tests (%)	Discovery/release (breeding year)
Red-fleshed cultivars	1	R 201 Kissabel®	RF4	21 (40%)	21st century
	2	Redlove®	RL	25 (48%)	2010
	3	Red Moon®	RM	27 (52%)	21st century
	4	Baya Marisa®	BM	29 (56%)	21st century
	5	Y 103 Kissabel®	RF2	37 (71%)	21st century
Old cultivars	1	White Winter-Callville	WW	38 (73%)	16th–17th century
	2	Kanada Renette	KR	40 (77%)	18th century
	3	Tiroler Spitzlederer	TS	43 (83%)	19–20th century
	4	White Rosemary	WR	47 (90%)	18th–19th century
	5	Goldparmäne	GP	30 (97%)	16th century
New cultivars	1	Pink Lady® Rosy Glow	PL	41 (79%)	1970s
	2	Santana	SA	42 (81%)	1996 (1978)
	3	Lb17906	LB	43 (83%)	2013 (1999)
	4	Bonita	BO	44 (85%)	21st century
	5	Gloster	GO	45 (87%)	1960s
	6	Fuji Zhen®	FJ	46 (88%)	1960s (1930s)
	7	CIVG 198 Modi®	MO	46 (88%)	21st century
	8	SQ159 Natyra®	NA	46 (88%)	2016 (1990s)
	9	Topaz	TO	47 (90%)	1990s (1984)
	10	Golden Delicious Klon B	GD	49 (94%)	19th century
	11	Bay 4210 Sonnenglanz®	SG	49 (94%)	21st century
	12	Gala Buckeye®	GA	50 (96%)	1965 (1934)
	13	Elstar Lb® 87/1	EL	30 (97%)	1975 (1955)

Prick reactions (>3 mm) of the 23 tested red-fleshed, old and new apple cultivars are shown including their time of discovery or release, modified after Vegro *et al.*, 2016 (n = 52, 100%, except EL, GP which were tested solely in Austria, n = 31, 100%).

Skin prick tests

The skin tests were performed on the flexor surface of the forearm by using the prick-to-prick procedure according to Heinzerling *et al.*[23,24] (Fig. 1). All tests were completed outside the birch pollen season. Each test consisted of 12-14 different apple cultivars (comprising flesh, peel equatorial, peel apical), birch pollen extract, histamine as a positive control and diluent as a negative control. Antihistamines or other test interfering medications

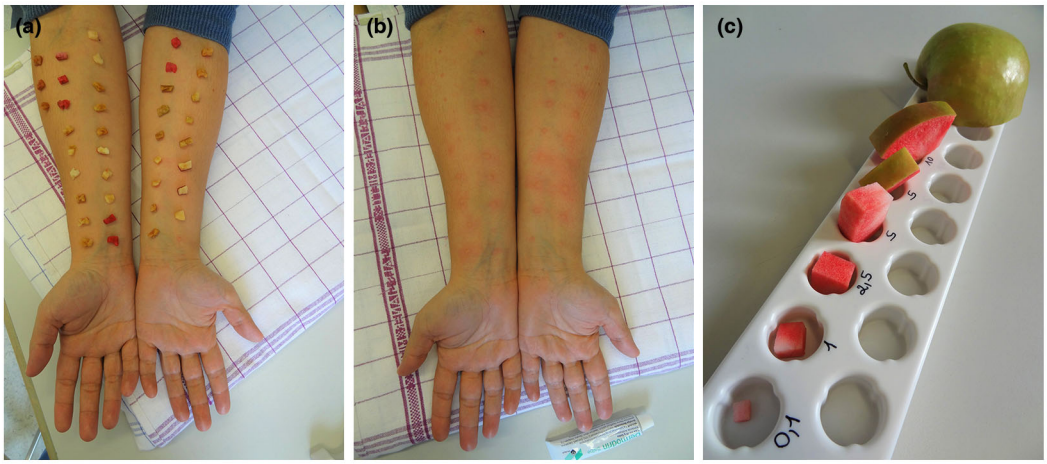

Figure 1 Allergenicity assessment. (a, b) Prick-to-prick test with apples. (c) Oral provocation test with Red Moon®.

were stopped at least 1 week before testing. Reproducibility of the test was determined by testing the reaction of the lowest and highest allergenic apple cultivar a second time on a different place of the forearm.

Oral provocations

Three oral provocation tests with one low, middle and high allergenic apple cultivar (Red Moon®, Pink Lady®, Golden Delicious respectively) were performed. The cultivars were chosen based on the results of skin reactivity. Apples were grown in South Tyrol (IT), harvested at physiological ripening stage, stored under standard commercial conservation conditions until use and sliced and prepared within 30 minutes before administration. Thirty-three participants (19 Austrian, 14 Italian) took part in the oral provocation tests, whereby six participants omitted one or two OPTs. All tests took place outside the birch pollen season. A break of at least 2 days was induced between each challenge. OPT has been modified after Nybom *et al.*[25] and Kopac *et al.*,[7] so increasing amounts of allergenic apple pieces were given to patients to consume (Fig. 1). The first portions (0.1, 1.0, 2.5, 5.0 g) were peeled fruit flesh, given in a five-minute interval. After that, patients started to eat unpeeled fruit starting with 5, 10, 20 and 40 g. Afterwards, the dose was doubled (80 g) in a 30-minute interval and further increased by 100 g until a maximum dose of more than one full apple was reached. Subjective symptoms were scored on a visual analog score (VAS) with a range of 0–10, where 0 is equal to no symptoms, 1–2 means mild symptoms, 3–5 moderate symptoms and > 5 represents severe symptoms. The provocation was stopped after moderate or severe symptoms occurred or when the patient wanted to stop the challenge. Reactions (e.g. itching or scratching, swelling of lips and difficulty swallowing or shortness of breath) were noted after each dose, and a final assessment was conducted 30 minutes after the last intake.

Statistics

Demographic (e.g. age) and ImmunoCAP results are expressed as median including ranges or quartiles (25th–75th). SPT results are shown as HEP-Index diameter (allergen average weal diameter divided by the positive control average diameter). Data were not normally distributed (Shapiro–Wilk test); non-parametric tests were therefore used. The Spearman rank test was used to determine correlations. The Wilcoxon signed-rank test was applied to compare red- and white-fleshed or old and new apple cultivars. Differences between the apple parts (flesh, peel and stalk) were determined with the Kruskal–Wallis test including subsequent Post hoc tests (Dunn–Bonferroni correction). A Friedman two-way ANOVA and Post hoc tests were used to analyse differences between the 23 cultivars. A p-value < 0.05 was regarded as statistically significant. All statistical analyses were performed with SPSS 25.[26]

Results

Demographics

The 52 participants included in this study had a median age of 40 years (range, 22–68), and 67% were female. All participants displayed IgE specific for Bet v 1 (Median 11.75 kUA/L; range, 2.9–69.00 kUA/L) and Mal d 1 (Median 3.34 kUA/L; range, 0.44-25.00 kUA/L). Bet v1 showed a positive correlation to Mal d1 (Spearman's rho = 0.7, $P < 0.001$) and with the following apple SPT results in the next paragraph (rho = 0.5, $P < 0.001$).

Skin prick tests

Twenty-three different apple cultivars, split into the apple parts flesh, peel and stalk, were tested. To evaluate the reproducibility of SPTs, lowest and highest apple skin reactions were retested. Only a low deviation of on average 1 mm mean weal diameter was found. Further, fast browning of apple cubes within five minutes after thawing for the cultivars White Rosemary, White Winter-Calville, Gloster and Kanada Renette was observed. Conversely, no browning was observed for red-fleshed apples.

During skin prick testing, differences in allergenicity were detected both within (flesh, peel and stalk) and between red-fleshed, old and new apple cultivars, Fig. 2. Nonetheless, the ranking of the single cultivars remained the same if sorted by the parts flesh, peel and stalk. In all participants, red-fleshed apples induced lower skin reactions in comparison with white-fleshed apples ($P < 0.001$, $r = 0.829$). They induced no skin reaction in 46.6% of the participants. Within the white-fleshed apples, especially old apple cultivars (Kanada Renette, Tiroler Spitzlederer and White Winter-Calville) but also Santana and Pink Lady® were lower in skin reactivity with 22.4% and 20.2% negative SPTs than other

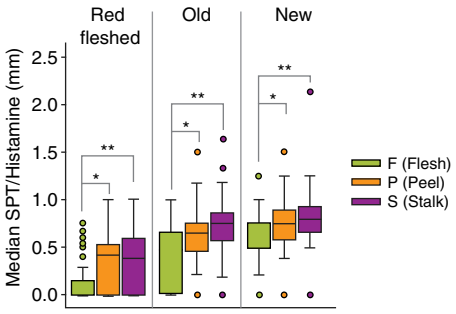

Figure 2 SPT results of red-fleshed, old and new cultivars. The allergenicity of the apple parts flesh, peel and stalk of 52 tested participants is given in median SPT/histamine (mm); significant differences between flesh–peel (F-P), flesh–stalk (F-S) and peel–stalk (P-S) are shown *$P < 0.05$, **$P < 0.001$, Kruskal–Wallis test and Post hoc test with Bonferroni correction.

commercial cultivars. Gloster, Lb 17906, Modi®, Fuji Zhen®, Elstar, Topaz, Sonnenglanz®, Natyra®, Golden Delicious and Gala, in fact, showed 9.9% or less negative SPTs ($P < 0.001$, $r = 0.813$). Interestingly, the sorts White Rosemary and Goldparmäne showed higher SPT responses in comparison with other tested old apple cultivars displaying 93.5% positive reactions. A more precise cultivar ranking including birch pollen is illustrated in Fig. 3 and Table 1. Apple cultivars with a HEP-Index diameter lower than 0.5 showed no significant intragroup differences ($P = 1.0$) but were significantly different from apple cultivars with a HEP-Index higher than 0.7 ($P < 0.05$), which also displayed no intragroup differences within their group ($P = 1.0$). Based on these results, apple cultivars were divided into low allergenic (HEP-Index < 0.5), middle allergenic (HEP-Index 0.5–0.7) and high allergenic (HEP-Index > 0.7).

Further analysis of the individual apple parts revealed differences in 16 apple cultivars ($P < 0.001$). As may be seen in Fig. 2, skin reactivity increased from flesh to peel and to stalk. A striking increase in skin response from flesh to peel was especially found for old apple varieties. In almost 70% of the participants, peel thereby induced higher responses than flesh. For red-fleshed apples, no differences between peel and stalk were found.

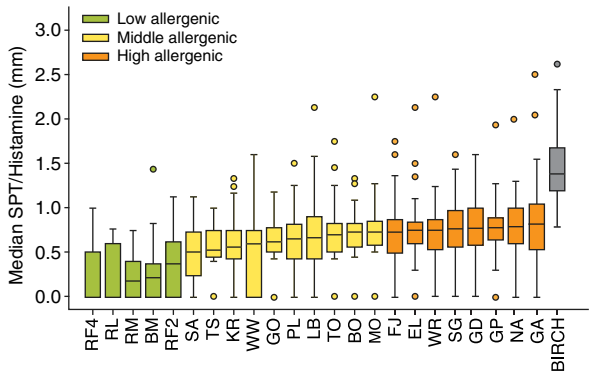

Figure 3 Apple prick-to-prick test (SPT) with 23 apple cultivars including birch, median SPT of flesh–peel–stalk/histamine (mm) [HEP-Index]. Twenty-one cultivars were tested in Austria and Italy ($n = 52$), EL and GP solely in Austria ($n = 31$). The apples are ranked by increasing allergenicity and divided into three groups (low, middle and high allergenic) based on pairwise comparison using the Friedman test with Dunns post-test, $P < 0.05$. The low allergenic group (HEP-Index < 0.5) showed no intragroup differences and was significantly different from the high allergenic group (HEP-Index > 0.7) which also displayed no statistically significant intragroup differences. BM, Baya Marisa®; BO, Bonita; EL, Elstar; FJ, Fuji®; GA, Gala®; GD, Golden Delicious; GO, Gloster; GP, Goldparmäne; KR, Kanada Renette; LB, Lb 17906; MO, Modi®; NA, Natyra®; PL, Pink Lady®; RF2, Red-fleshed 2; RF4, red-fleshed 4; RL, Redlove®; RM, Red Moon®; SA, Santana; SG, Sonnenglanz®; TO, Topaz; TS, Tiroler Spitzlederer; WR, Weißer Rosemary; WW, White Winter-Calville.

Oral provocations

The participants were orally challenged with increasing doses of Red Moon®, Pink Lady® and Golden Delicious, as shown in Fig. 4. SPT results predicted the severity of OAS of each apple cultivar. Further a significant negative correlation between the SPT and OPT results between all apples (rho = -0.4, $P < 0.01$) and especially between the lower allergenic Red Moon® apple was found (rho = -0.5, $P = 0.01$). Apples with high reactivity in SPT also triggered increased symptoms even in small amounts during OPT; hence, less could be eaten. As a summary, participants were able to eat significantly more of red-fleshed apples than of white-fleshed apples ($P < 0.05$). Most of the patients' complains were about itching or scratching in the mouth and throat; however, in some cases, also a running itching nose, watery itching eyes and itching of the ears were noticed. Only a few patients recognized swelling of the throat and hence difficulty in swallowing or shortness of breath. All side-effects resolved spontaneously within 30 min.

Red Moon® In 49% of the patients, unpeeled pieces in an amount of 13.6–163.6 g induced first oral symptoms, whereby 29% of the participants were able to eat more than one full apple without any symptoms.

Pink Lady® Peeled Pink Lady® already induced first symptoms at a range of only 0.1–8.6 g in 54% of the cases. About 36% reacted after eating unpeeled samples in a range between 13.6–

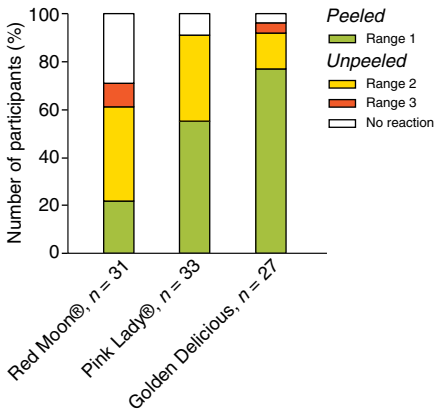

Figure 4 Number of participants (%) reacting on different amounts of apple. Low, middle and high allergenic apple sorts RM, PL and GD were tested by oral challenges. The respective apple amounts that induced first OAS symptoms (e.g. itching/scratching) are divided into three ranges: range 1 (0.1–8.6 g) peeled, range 2 (13.6–43.6 g) and range 3 (83.6–163.6 g) unpeeled. In some cases, more than one full apple (163.6 + 100 g) was tolerated without any symptoms (=no reaction).

43.6 g. Only three of the participants were able to eat one full apple.

Golden Delicious This cultivar showed OAS in already small quantities. More than 78% reacted after eating 0.1-8.6 g peeled apple. Only two patients could eat 80 g or a whole apple without OAS.

Discussion

In this study, we analysed the allergenic potential of 23 apple cultivars to find suitable varieties for AIT to birch and pollen-related apple allergy and provide an allergenicity ranking shown in Fig. 3 according to the SPTs. As hypothesized, our participants responded less strongly or even not at all to red-fleshed and traditional old apple cultivars. Some commercial sorts were nonetheless also well tolerated and therefore fitting candidates for starting AIT.

To our knowledge, this is the first study that systematically analysed skin reactivity and OAS of red-fleshed cultivars. The reddish apples showed the weakest reactions in SPT and induced no or very mild symptoms during OPT, suggesting red-fleshed apples as optimal 'starter' apples for AIT. As Mal d 1 concentrations increases with duration of storage,[22,27,28] lower allergenicity in cultivars with less storability can be further explained with a lack of time that the cultivars need to establish higher allergen quantities during conservation. Old varieties have already been described as rather well tolerated, but to date, only a few varieties have been analysed in prick tests.[17,29–31] A recent study of Vegro *et al.*[17] showed low allergenicity of the old sorts Tiroler Spitzlederer and Calvilla Bianca d'Inverno (i.e. White Winter-Calville). AIT with red-fleshed or well-tolerated old cultivars as Kanada Renette, Tiroler Spitzlederer and White Winter-Calville can best be started in autumn when apples are freshly harvested, since storability of these cultivars is limited. After the 'starter' apples, therapy must be continued in winter with commercial cultivars with longer storability and higher Mal d1 content also to improve the treatment effect. Apart from AIT, peeled old cultivars and red-fleshed apples can be recommended for consumption in patients with birch prFA; however as interindividual differences exist, we recommend starting with a small peeled peace, to avoid strong OAS reactions.

As mentioned before, new and especially commercial varieties like Pink Lady®, Fuji, Gala or Topaz were specially bred aiming increased storage properties, more resistance and better taste and past studies, as well as ours, indicate that this increased the allergenicity of cultivars. Indeed, six of the 13 tested new cultivars induced high and further six moderate SPT reactions. Thereof, Santana induced lowest reactions which confirm previous findings.[16,18,27,32] Also, Pink Lady® seems to be better tolerated than other commercial cultivars. This is supported by previous findings where Pink Lady® showed low Mal d 1 content and less skin reactivity.[17,33] Golden Delicious, a well-known apple with high allergen content, triggered strong reactions, also in our study,[12,13,15,17,33,34] as did Gala, which induced the highest SPT reactions of all tested cultivars. Also, the cultivars Natyra®, Sonnenglanz® and Fuji showed significantly high SPT reactions and the finding of high Mal d 1 content in previous research is in line with our study.[13,15,17,21,33–36] High allergenic apples should be used for AIT maintenance dose after less allergenic varieties have been tolerated during a build-up phase.

Based on the results of our study and in accordance with the protocol described by Kopac *et al.*,[7] we propose AIT with apples with a minimum duration of least 8 months, which is tested in an ongoing study.

Treatment is started with a low allergy apple according to Fig. 3, e.g. freshly harvested red-fleshed apple cultivars like Red Moon®, an old cultivar selected by individual SPT reactivity or Santana. Starting dose is the largest amount tolerated in the preceding OPT as described in the Method section. The patient should chew the portion of apple carefully for 2 min leaving some amount sublingually. Food and water intake should then be avoided for minimum of 15 min thereafter. If the current amount is well tolerated, the allergen concentration is increased over the next month's doubling the apple intake every 2 weeks until a maximum dose of one full apple is reached. Thereafter, the cultivar is replaced by a medium or moderate allergenic commercial apple cultivar for another 6 weeks like Pink Lady®, Gloster or Topaz; then, a switch to a high allergenic cultivar should be attempted with, e.g., Gala, Natyra, Goldparmäne, Golden Delicious or Sonnenglanz. The respective cultivar with high allergic properties will be consumed in the maximum tolerated dose for the last 5 months of AIT, and thereafter, if acceptable. Changing of the apple cultivar is pretested by OPT to find the suitable daily starting amount as described in the method section. In the ongoing treatment study IgE and IgG scores, conjunctival provocation tests and birch pollen-related symptoms will be analysed before and after for AIT assessment.

Herein, we propose a treatment protocol ready for the use of allergen immunotherapy to birch pollen and birch prFA to apples with daily apple consumption. Red-fleshed, old and the new cultivars Santana and Pink Lady® provoke less allergic reactions and are suitable for initial induction. After a controlled tolerated increase of intake, also moderate and finally high allergenic apple cultivars are consumed. Peeled old and red-fleshed cultivars can be recommended for consumption in patients with birch prFA who do not seek treatment and wish to consume apples.

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