Clinical Study of Pollen-food Allergy Syndrome Estimated by Double-blind, Placebo-controlled Food Challenges of Ten Apple Cultivars

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Background : The number of patients with pollen-food allergy syndrome (PFAS) has increased globally. Apples are a major causative food for PFAS. Varied reports have demonstrated the usefulness of skin prick test (SPT) for the diagnosis of PFAS, besides differences in symptom expression among apple cultivars, thus the diagnosis of PFAS remains unclear.

Purpose : To investigate the clinical features of apple-induced PFAS by performing a double-blind placebo-controlled food challenge (DBPCFC) using different apple cultivars on patients with apple-induced PFAS.

Method : DBPCFC was performed for six patients with apple-induced PFAS using 10 apple cultivars. We measured the degree of symptoms using the Visual Analog Scale (VAS). Further, we assessed the correlations of Mal d 1 and Bet v 1-specific IgE levels and SPT findings to the VAS.

Results : Three of six patients (50 %) were VAS-positive for two apple cultivars, one patient each (17 %) was positive for three, four, and five apple cultivars. The correlation between SPT findings and VAS was insignificant ($p = 0.103$). The VAS displayed a positive relationship with Mal d 1 and Bet v 1 ($r = 0.5$ and $r = 0.84$, respectively).

Conclusion : It is necessary to perform DBPCFC with multiple apple cultivars to diagnose PFAS. The SPT was not useful in diagnosing PFAS ; however, Bet v 1-specific IgE levels may be advantageous. This novel clinical study of PFAS assessed multiple Japanese apple varieties, therefore, our findings can serve as a baseline for future studies. *Shinshu Med J 71 : 99—107, 2023*

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Key words : pollen-food allergy syndrome, skin-prick test, visual analog scale, oral food challenge

I Introduction

The number of patients with hay fever has increased worldwide, including Japan[1]. This increase includes an upsurge in the number of patients diagnosed with pollen-food allergy syndrome (PFAS)[2)-4)].

Apple, a frequently consumed fruit, is a major trigger allergen for PFAS. The typical symptoms of PFAS vary from oral and throat mucosal symptoms to birch pollinosis. Mal d 1 is the causative protein of allergy symptoms in patients with apple-induced PFAS ; it induces a reaction by cross-reactivity with Bet v 1, the major birch pollen allergen[5)-8)]. PFAS often develops in school-age children and adults, and their quality of life gets impaired because they are unable to eat frequently consumed foods ; it is difficult to expect a spontaneous remission[9)-13)].

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The diagnosis of PFAS is difficult because the symptoms are predominantly oral, besides a lack of objective symptoms. Skin prick tests (SPTs) are generally considered useful for diagnosing PFAS[14]. However, SPTs alone may not be diagnostic in actual clinical practice and some reports reveal that there is a relationship between SPT and PFAS symptoms[15]. Clinicians need to perform an oral food challenge (OFC), which entails the ingestion of an allergen-containing food, to observe symptom occurrence[2]. Because the symptoms of PFAS are subjective, a single-blind OFC may generate false-positive results in the presence of psychogenic thus, lacks diagnostic accuracy[16]. Further, few reports elucidate a double-blind placebo-controlled food challenge (DBPCFC) comprising numerous apple varieties[15][17]. Apple varieties differ in their antigenicity[17]-[19] ; therefore, DBPCFC should be performed on several apple varieties for an accurate diagnosis.

Thus, we aimed to perform DBPCFC on 10 of the 28 apple varieties whose Mal d 1 mRNA expression levels were analyzed by our coauthors[20] and whose antigenicity was expected to vary based on the results. Further, we intended to examine the relationship between OFC results and Mal d 1- and Bet v 1-specific IgE levels.

Ⅱ Methods

A Study participants

We recruited patients who visited the Nagano Children's Hospital between September 2019 and January 2020, had a known allergy to raw apple, and tested positive for birch pollen-specific IgE (≥0.35 kUA/L). Six participants (all female, median age 24.5 years, range 10-45 years) who had previously provided written informed consent were included in this study.

B Double-blind placebo-control food challenge (DBPCFC)

The DBPCFC was conducted during the hay fever season (September 2019 to January 2020) characterized by a good supply of apples in the market. We used 10 apple cultivars from the Nagano Prefecture, including the Shinano-piccolo (SP), Shinano-dolce (SD), Sun-Tsugaru (ST), Akibae (Aki), Ourin (O), Sennshu (Se), Toki (To), Shinano-hoppe (SH), Aika no kaori (AK), and Sun-fuji (SF). The DBPCFC consisted of apple and cabbage smoothies, which followed the protocol of the Model II pita bread study by Hansen et al.[16], such that the protocol could be applied to patients with wheat allergies as well. The smoothies were prepared using 25 g of apples, 50 g of cabbage, 35 ml of apple juice, 2.5 ml of lemon juice, and two teaspoons of sugar. Based on the Japanese style of apple consumption, the apple peels were removed before preparing the smoothie. To mask the taste, we added commercially processed apple juice (trade name : Apple Clear, Harada Dairy) with inactivated Mal d 1. The placebo was prepared using similar ingredients as the apple smoothie, but without adding fresh apples. The patients ingested four to five smoothies (three–four apple smoothies and one placebo smoothie) per OFC. OFCs were conducted thrice across different days to permit the patients to be challenged with 10 apple cultivars. For patients under antihistamine medications, we discontinued the medication 3 days before performing the OFC. Co-investigators who were not involved in the OFC randomized the smoothies in the food challenge. The interval between the ingestion of each smoothie was ≥ 30 min to ensure sufficient time for the symptoms to disappear in participants who developed them after ingesting a smoothie. The smoothies were prepared immediately before the OFC. If the DBPCFC could not be conducted on a similar day, the apples were stored in a freezer at −18℃ to ensure consistency in the Mal d1 content[21].

We used the Visual Analog Scale (VAS), which measures the intensity of clinical symptoms, to evaluate the intensity of symptoms experienced by each patient ; the intensity ranged from 0 (none) to 100 (strongest). The patients were provided a sheet of paper with a 100 mm line drawn on it : the left end represented "no symptoms (=0)," whereas the right end represented "worst symptoms (=100)." The patients were requested to indicate their degree of pain on that line upon exhibiting symptoms. The rater assessed the results and assigned them a numerical

value. OFC positivity was defined as a VAS＞0.

C Skin prick test (SPT)

The SPT was performed on the volar side of the forearm using a bifurcated needle (Tokyo MI Company, Tokyo, Japan) and the prick-to-prick method. We performed SPTs on the day of the OFC and the day before. Histamine dihydrochloride (10 mg/ml) (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) was used as a positive control, whereas a scratch extract (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) was used as a negative control solution. We measured wheal diameter 15 min following pricking. A positive result was defined as a wheal diameter ≥3 mm than that of the negative control[22)23)].

D Laboratory Data

We measured the specific IgE to apples, birch pollen, Mal d a, Mal d 3, and Bet v 1 using the ImmunoCAP® assay system (Thermo Fisher Scientific/Phadia, Uppsala, Sweden). The specimens were collected on the day of the first OFC and the day before. A positive result was defined as an IgE level ≥0.35 kU/l. The OFC and SPT results were compared to determine the usefulness of the SPT for diagnosing PFAS.

E Statistical analyses

We performed the Chi-square test to compare the number of patients with a positive VAS score and those who were SPT positive. The Spearman's rank correlation coefficient was used to evaluate the specific IgE levels, VAS, VAS positivity rate, and Mal d1 levels. The level of statistical significance was set at a p-value ＜0.05 (one-tailed). GraphPad Prism 9 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses. The age and specific IgE values are presented as the median (range).

F Ethical considerations

This study was approved by the Nagano Children's Hospital Institutional Review Board (Approval No.: 31-28, Approval date: September 03, 2019). All study participants or their legal guardians provided written informed consent.

Ⅲ Results

A Study participants

Six patients with apple-induced PFAS participated in this study. **Table 1** summarizes their characteristics. All patients were female, with a median age of 24.5 (10-45 years) years. All patients had allergic rhinitis

Table 1 Characteristic of the study participants

Case		1	2	3	4	5	6	Median
Sex		F	F	F	F	F	F	
Age (y)		10	12	14	35	42	45	24.5
Age at onset of PFAS (y)		9	7	10	12	14	14	11
Allergic factors		AR	AR	AR	AR	AR	AR	
Specific IgE	Birch pollen (U_A/ml)	34.2	＞100	7.62	3.82	2.33	3.75	5.72
	Cedar pollen (U_A/ml)	10.1	87.4	37.4	9.16	0.98	9.66	9.41
Foods other than apples that cause PFAS			peach, kiwi fruit, cherry, tomato, strawberry, loquat, pear, orange	peach, cherry, pear	peach, cherry, pear, grape		peach, kiwi fruit, cherry, strawberry, loquat, pear	

AR ; allergic rhinitis ; IgE, immunoglobulin E ; and PFAS, pollen-food allergy syndrome

Table 2 Specific IgE and VAS of DBPCFC

Case	Specific IgE			VAS of DBPCFC											
	Mal d 1 U_A/ml	Mal d 3 U_A/ml	Bet v 1 U_A/ml	SP	SD	ST	Aki	O	Se	To	SH	AK	SF	Positive rate (%)	mean VAS score
1	13.3	<0.1	26.2	90	0				90	0				50	90
2	90.9	<0.1	>100	0	0	0	85	0	50	5	0	25	0	40	41.3
3	1.21	<0.1	2.61	15	35	0	0	0	0	0	0	0	0	20	25
4	0.85	<0.1	2.79	10	0	10	10	0	5	15	0	0	0	50	10
5	2.29	<0.1	5.97	0	0	0	10	5	0	0	0	0	0	20	7.5
6	3.34	<0.1	4.59	0	0	0	10	10	0	10	0	0	0	30	10

SP, Shinano-piccolo ; SD, Shinano-dolce ; ST, Sun-Tsugaru ; Aki, Akibae ; O, Ourin ; Se, Sennshu ; To, Toki ; SH, Shinano-hoppe ; AK, Aika no kaori ; SF, Sun-fuji ; IgE, immunoglobulin E ; DBPCFC, double-blind placebo-controlled food challenge ; and VAS, Visual Analog Scale.

as a comorbidity. The patients were positive for cedar pollen, with a level of 9.41 (0.98-87.4) U_A/ml. Four of the six patients consumed PFAS-causing foods other than apples. None of the patients had consumed any antihistamines for 3 days prior to the OFCs.

B DBPCFC (VAS) and specific IgE levels

Table 2 summarizes the DBPCFC results (VAS) and specific IgE levels of each patient for birch and apple. Patient 1 could not undergo all food challenge tests because of the symptom intensity ; the patient participated in the OFCs for four apple cultivars (SP, SD, Se, and To). All patients were VAS-positive for at least two apple cultivars and VAS-negative for several others. All patients were VAS-negative for the placebo. They were Mal d 3-negative **(Table 2)**. The median Mal d 1 level was 2.82 (0.85-90.9) kU/l, whereas the median Bet v 1 level was 5.28 (2.61->100) kU/l. We observed a correlation between the Mal d 1- and Bet v 1-specific IgE levels ($r = 0.89$ and $p = 0.024$).

Table 2 depicts the VAS positivity rate according to the apple cultivars. The VAS positivity rates were 50 % (3/6) for SP, 17 % (1/6) for SD, 20 % (1/5) for ST, 80 % (4/5) for Aki, 40 % (2/5) for O, 50 % (3/6) for Se, 50 % (3/6) for To, 0 % (0/5) for SH, 20 % (1/5) for AK, and 0 % (0/5) for SF **(Table 2, Fig 1)**.

1. Relationship between specific IgE levels and the VAS

We observed a moderate positive correlation between the Mal d1-specific IgE levels and the mean VAS score for the apples for which the patients tested OFC-positive ($r = 0.5$ and $p = 0.27$) ; however, the correlation was insignificant **(Fig. 1)**.

Meanwhile, we identified a significant positive correlation between the Bet v1-specific IgE levels and the mean VAS values for the apples for which the patients tested OFC-positive ($r = 0.84$ and $p = 0.09$) **(Fig. 2)**.

C Correlations between SPT and VAS

Fig. 3 depicts the relationship between the SPT results and VAS scores according to the apple cultivars.

ST and To with large wheal diameters ranging from 7 mm to 9 mm had low VAS scores ranging from 0 to 5, Se, Aki, and SP with wheal diameters ranging from 3 to 5 mm had high VAS scores ranging from 85 to 90, thus indicating the overall variations. We observed VAS positivity and SPT positivity in 12/18 subjects (VAS-positive and SPT-positive/VAS-positive, 67 %), whereas VAS negativity and SPT negativity were observed in 21/37 subjects (VAS-negative and SPT-negative/VAS-negative, 57 %) ($p = 0.103$).

Ⅳ Discussion

In this study, patients with apple-induced PFAS underwent DBPCFC of 10 different apple varieties. Previous studies performed single-blind OFC on pa-

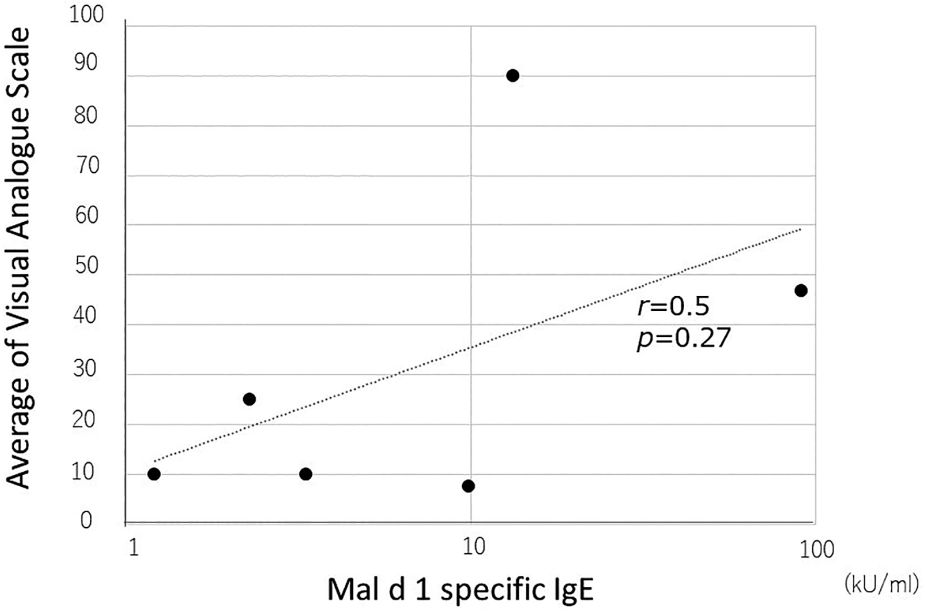

Fig. 1　Correlation between Mal d1-specific IgE levels and the average Visual Analog Scale (VAS) in patients who are oral food challenge-positive to the apple cultivars

The horizontal axis denotes the log Mal d1-specific IgE level, whereas the vertical axis denotes the average VAS. The straight line that rises steadily to the right is the regression line. A positive insignificant correlation has been observed ($r = 0.5$, $p = 0.27$).

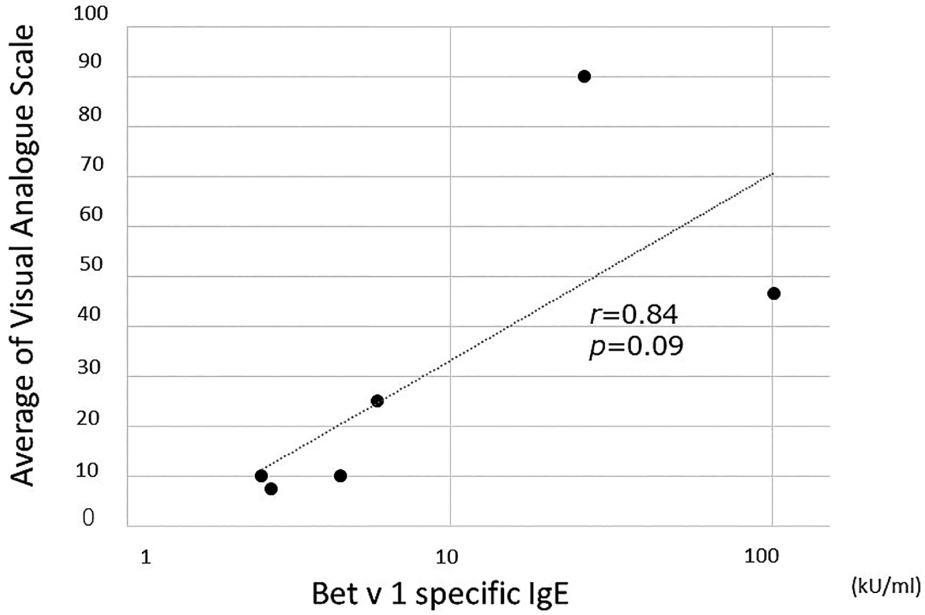

Fig. 2　Correlation between Bet v1-specific IgE levels and the average Visual Analog Scale (VAS) in patients who are oral food challenge positive to the apple cultivars

The horizontal axis denotes the log Bet v1-specific IgE levels, whereas the vertical axis denotes the average VAS. The straight line that rises steadily to the right is the regression line. A significant and positive correlation has been observed ($r = 0.84$, $p = 0.09$).

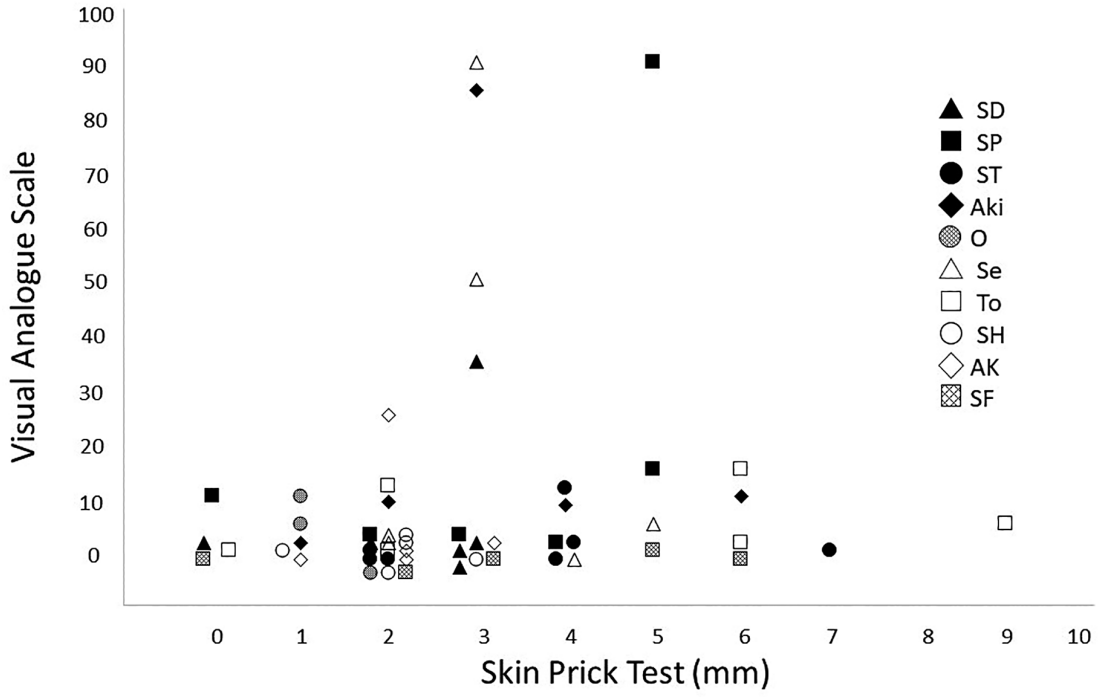

Fig. 3 Correlations between the skin prick test (SPT) and the Visual Analog Scale (VAS)

The wheal diameter (mm) resulting from the SPT is presented on the horizontal axis, whereas the VAS is presented on the vertical axis. Twelve/18 types are VAS-positive and SPT-positive (VAS positive and SPT positive/VAS positive, 67 %), whereas 21/37 types are VAS-negative and SPT-negative (VAS negative and SPT negative/VAS negative, 57 %).

SP, Shinano-piccolo ; SD, Shinano-dolce ; ST, Sun-Tsugaru ; Aki, Akibae ; O, Ourin ; Se, Sennshu ; To, Toki ; SH, Shinano-hoppe ; AK, Aika no kaori ; and SF, Sun-fuji ;

tients with PFAS[15]. However, PFAS is primarily a subjective symptom, and single-blind studies are not reliable for determining the presence of symptoms with OFC. This necessitates modifying the morphology of apples to perform DBPCFC. Limited studies have focused on DBPCFC using apples, all of which were conducted in Europe[16)17)]. Hansen et al.[16] conducted a study by hiding apple pieces inside pita bread and adding cabbage to mask the texture of the apples. However, patients with wheat allergies cannot consume pita bread, thus, we performed the DBPCFC using apple smoothies following this recipe. Furthermore, the conversion of apples into smoothies ensured that the apples did not maintain their original form such as a pita bread, thus creating a higher mask effect. Suzanne et al. mixed apples and yogurt to prepare the challenge food in their study. Dairy allergies are the second most prevalent allergies in Japan[24]. Conversely, the smoothies used in this study were less likely to contain allergens ; our recipe can be used as a reference for DBPCFC in the future. In this study, OFC-positive and OFC-negative cultivars differed among the participants. However, the reliability of our study is supported by the DBPCFC approach instead of a single-blind approach and the absence of symptoms with placebo ingestion. Therefore, this study demonstrated the potential of validity of DBPCFC using apple smoothies.

Scattered reports have used DBPCFC for patients with apple-induced PFAS in Europe, nonetheless these reports used only two to four apple varieties[15)17)]. In the present study, we performed DBPCFC on numerous apple varieties[15] selected from the co-authors' previous report[20] and evaluated it using a more rigorous approach. Previous results have revealed differences in symptom appearance among apple varieties. Vlieg-Boerstra et al. used four apple cultivars[15] in an OFC. Despite a significant difference in the

symptom intensity, the VAS score (range : 0–80) varied significantly according to the cultivar. An OFC for the diagnosis of apple-induced PFAS is usually performed on a single variety, however, the results of Vlieg-Boerstra et al. suggested that an OFC on fewer varieties may generate false-negative results or even the removal of other varieties from the diet that can be consumed without symptoms, despite being OFC-positive. Further, considering no difference in VAS for each of the original apple variety crosses[20], it is necessary to study more cases of individual apples.

The mechanism of PFAS is due to IgE cross-reactivity between fruit allergens and pollen allergens. First, the patient is sensitized to pollen, following which fresh fruits and vegetables come into contact with the sensitized site, the oral mucosa, causing a type I allergic reaction. However, the fruit allergens involved in PFAS are unstable to digestion, thus, lose their antigenicity during digestion, resulting in fewer organ symptoms outside the oropharynx[25)-27)]. Bet v 1-related protein is a type of infection-specific protein produced by plants to protect themselves from physical stresses such as invasion by infectious microorganisms and insect damage. It is also found in birch pollen[27]. It is cross-antigenic with Bet v 1, the major allergen of birch pollen, and Mal d 1, an allergen found in apple[13]. In Europe, Mal d 3 reportedly cause systemic symptoms as the causative protein pf apple-induced PFAS, unlike Mal d 1. In the present study, Mal d 3-specific IgE levels were measured prior to OFC[1)27)28)], and all patients were negative, thus allowing a safe OFC. We observed an insignificant but positive correlation between Mal d 1-specific IgE levels and VAS, and a significantly positive correlation between Bet v 1-specific IgE levels and VAS, thereby suggesting Bet v 1-specific IgE levels may be useful for the diagnosis of apple-induced PFAS. Our results are highly novel because few studies have examined the correlation between specific IgE levels and VAS in patients with DBPCFC, despite several reports on the correlation between SPT and VAS.

Our results revealed that SPT was not useful for the diagnosis of PFAS, despite being considered generally useful for the same[29]. The OFC results were consistent with SPT results in another report[6]. The present study, as well as previous reports that mentioned the SPT is not useful for the diagnosis of PFAS[15)30)], indicated that the SPT cannot be used to make a definitive diagnosis of PFAS. The results of the present OFC on numerous apple varieties suggested that the SPT is unlikely to be a criterion for the diagnosis of PFAS in the future, besides the need to incorporate the use of an OFC with multi-cultivar apples.

This study had some limitations. First, it had a small sample size. PFAS often manifests in children after school age. This study comprised 10 apple cultivars, thus, the patients were admitted for several days to undergo assessments, which suggested that several patients with PFAS could not participate because of school or work. Despite a positive correlation between Bet v 1-specific IgE levels and VAS in six cases, we included outliers in case 1. We observed a weak positive correlation ($r = 0.58$, $p < 0.001$), excluding this outlier and examining the five cases. Researchers should investigate the correlation between Mal d1 levels and VAS scores in a larger patient sample. The absence of difference in VAS for each of the original apple variety crosses warrants investigating higher cases of individual apples. Currently, we are conducting a study with additional cases and will report its results in the future. Second, the reproducibility of the OFC has not been completely established. Bet v 1 may be a predictor of symptom appearance in apple PFAS. However, it is difficult to predict the difference in symptom appearance among different apple varieties in each case. In this study, patients with apple-induced PFAS could consume several apple varieties without symptom appearance, which necessitates, further studies on higher apple varieties, including a similar variety from different harvest years and other apple varieties.

V Conclusion

In summary, the DBPCFC was more accurate than a single-blind OFC in case of subjective PFAS symp-

toms. However, DBPCFC is labor intensive. The SPT was not useful in the diagnosis of apple-induced PFAS ; however, Bet v 1-specific IgE levels may be considered a diagnostic aid. The results obtained by performing DBPCFC on multiple apple varieties revealed differences in the appearance of PFAS symptoms, which suggests the importance for examining against multiple varieties for improving the diagnostic accuracy of PFAS.

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