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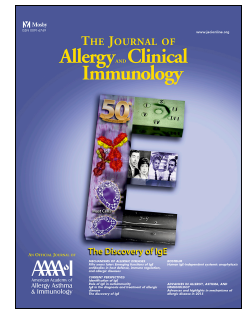
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Efficacy and safety of 4 months of sublingual immunotherapy with recombinant Mal d 1 and Bet v 1 in patients with birch pollen-related apple allergy

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ABSTRACT

Background: Birch pollen-related apple allergy is among the most prevalent food allergies in adolescent/adult individuals and mainly results from sensitization to the major birch pollen allergen Bet v 1 and subsequent cross-reaction with the apple protein Mal d 1. However, specific immunotherapy with birch pollen has inconsistent effects on apple allergy.

Objective: To compare the safety and efficacy of sublingual immunotherapy (SLIT) with two formulations containing either recombinant (r) Mal d 1 or Bet v 1 on birch pollen-related apple allergy.

Methods: Sixty participants with birch pollen-related apple allergy were randomized to daily sublingual application of placebo (n=20), 25 µg of rMal d 1 (n=20) or rBet v 1 (n=20) for 16 weeks. Adverse events were regularly recorded. Sublingual challenges with standardized doses of rMal d 1, skin prick testing with recombinant allergens and measurements of allergen-specific IgE and IgG4 antibodies were performed before and after treatment.

Results: Both formulations caused comparable, mainly local adverse events. No systemic reactions occurred. Compared to the placebo and the rBet v 1-treated group SLIT with rMal d 1 reduced rMal d 1-induced oral symptoms ($P=0.001$ and $P=0.038$), accompanied by longitudinally reduced rMal d 1-specific cutaneous reactions ($P=0.022$) and enhanced IgG4/IgE ratios ($P=0.012$). SLIT with rBet v 1 neither improved the clinical reactivity to rMal d 1 nor enhanced rMal d 1-specific IgG4/IgE ratios. Participants receiving placebo showed no allergen-specific changes.

Conclusion: Sublingual treatment with a recombinant food allergen was safe and clinically effective as determined by standardized challenges. We present a promising approach for the effective treatment of birch pollen-related apple allergy.

Clinical Implications

Daily sublingual administrations of a constant dose of recombinant Mal d 1 improve birch pollen-related apple allergy.

Key words: food allergy, birch pollen allergy, birch pollen-related food allergy, IgE, allergen-specific immunotherapy, recombinant allergens, Bet v 1, Mal d 1,

Abbreviations

Ab, antibody; AE, adverse events; AIT, allergen-specific immunotherapy; BP, birch pollen; BPE, birch pollen extract; BPRFA, birch pollen-related food allergy; cpm, counts per minute; OAS, oral allergy syndrome; r, recombinant; SCT, sublingual challenge test; SLIT, sublingual immunotherapy; SPT, skin prick testing;

Capsule summary

SLIT with recombinant Mal d 1 is a promising approach for the effective treatment of birch pollen-related apple allergy.

INTRODUCTION

Birch pollen-related food allergy (BPRFA) is the most prevalent food allergy in adolescent/adult individuals in Europe and also affects children¹. Typically, birch pollen-allergic patients suffer from an oral allergy syndrome (OAS) to certain foods, most often hazelnut, apple and peach. Handling these foods may also cause allergic cutaneous contact reactions and occasionally, their consumption induces worsening of atopic eczema and rarely anaphylactic reactions^{1, 2}. In contrast to respiratory symptoms which occur only during the birch pollen season, BPRFA usually persists perennially³. Consequently, BPRFA impairs the quality of life of around 70 million people worldwide. However, until now no efficient therapy for BPRFA exists.

BPRFA mainly results from primary sensitization to the major birch pollen allergen, Bet v 1, and subsequent immunological cross-reaction with Bet v 1-related food proteins, e.g. Mal d 1 in apple, Api g 1 in celery, Cor a 1 in hazelnut, and Gly m 4 in soy⁴⁻⁷. Hence, it was expected that the modulation of the Bet v 1-specific immune response by allergen-specific immunotherapy (AIT) concomitantly improves BPRFA. Indeed, some beneficial effects of birch pollen-AIT on BPRFA have been described⁸⁻¹². However, others have reported limited curative effects and in some cases even the onset of BPRFA during AIT¹³⁻¹⁸. We previously found that sublingual immunotherapy (SLIT) with birch pollen, i.e. applied directly at the site of food-induced allergic symptoms, improved birch pollinosis but failed to ameliorate apple-induced OAS¹⁹. On the other hand, daily consumption of gradually increasing amounts of apple was able to induce a transient oral tolerance in 17/27 (63%) apple-allergic patients²⁰. Beyond that, we observed immune responses indicative for peripheral tolerance development in birch pollen-allergic patients with apple allergy after sublingual administration of 50 µg of recombinant (r) Mal d 1 on two consecutive

days²¹. These promising findings inspired us to study the effects of SLIT with rMal d 1 on apple allergy and to compare it with rBet v 1.

We conducted a single centre, double-blind placebo-controlled explorative study including 60 birch pollen-allergic participants with apple allergy randomized to the daily sublingual administration of placebo, 25 µg of rMal d 1 or rBet v 1 (n=20 for each group) for 16 weeks. Adverse events (AE) were recorded regularly during the course of treatment. The efficacy on apple allergy was assessed with sublingual challenge tests (SCT) with standardized doses of rMal d 1 before and after SLIT²². The participants also underwent skin prick testing (SPT) with recombinant allergens, birch pollen extract and fresh apple. Additionally, allergen-specific IgE and IgG4 levels were monitored.

METHODS

Trial population

In 2012, we recruited 77 Austrian BP-allergic subjects aged 18-65 years who reported to experience apple allergy. These subjects displayed rhinoconjunctivitis in spring, positive SPT to birch pollen extract (BPE, ALK-ABELLO, Hørsholm, Denmark) and fresh apple (a typical local cultivar "Pink Lady"), and BPE-specific IgE levels of >0.35 kU/L (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden). We excluded subjects who experienced symptoms to perennial allergens or had received birch pollen-AIT within the past 5 years. After providing written informed consent 72 individuals were screened for apple allergy by means of sublingual challenge tests (SCT) with rMal d 1 in December 2012 - January 2013²². Four subjects declined to participate. Sixty participants (31 female, 29 male; median age 33 years, range 20-64 years) who responded with OAS in SCT to 1.6-25 µg of rMal d 1 were randomized 1:1:1 using the "Randomizer for clinical trials 1.8.1" (Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Austria; available at: <https://www.randomizer.at/>) to daily sublingual administration of rBet v 1 (25 µg/day), rMal d 1 (25 µg/day) or placebo (5 mM phosphate buffer, pH=7.4, supplemented with 25% v/v glycerol), respectively (Fig. 1).

Trial design

This randomized double-blind, placebo-controlled trial (ClinicalTrials.gov number NCT01449786 and EudraCT no 2011-001221-24) was conducted at the Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases (DIAID), Medical University of Vienna, Austria, in accordance with Good Clinical Practice, the Declaration of Helsinki and with ethical clearance of the local ethics committee (EK228/2011). The treatment period of 16 weeks started in August 2013. The first

administration of 300 µl of the study medication was performed at the DIAID. Participants were asked to keep the solution under their tongue for 1-2 minutes before swallowing. Each participant was monitored for the next 60 minutes and any adverse events (AE) were recorded. Subsequent doses were self-administered at home. Every four weeks, the subjects visited the DIAID to report AE that had occurred during the past four weeks. In addition, used glass flasks were exchanged for new ones containing the study medication for the following 4 weeks. The study was monitored by the Koordinationszentrum für Klinische Studien at the Medical University of Vienna, Vienna, Austria.

Investigational products

Bet v 1a-GMP-0802 (termed rBet v 1) and Mal d 1.0108-GMP-0902 (termed rMal d 1) were produced under GMP conditions by Biomay AG, Vienna, Austria. For sublingual administration, each allergen (84 µg/ml) was dissolved in previously determined optimum buffers (rBet v 1 in 5 mM phosphate buffer, pH=7.4; rMal d 1 in 20 mM carbonate buffer, pH=9.0), supplemented with 25% v/v glycerol and kept in dark glass flasks at 4°C. The concentration and integrity of both allergens under these conditions after storage at 4°C for six months was tested by means of bicinchoninic acid assay (BCA, Sigma Aldrich, Vienna, Austria), SDS-PAGE, ELISA, immunoblotting and size exclusion chromatography (see Table E1 and Figures E1-4 in the Online Repository) and proved stability of both study drugs.

Sublingual challenge tests

SCT were performed with increasing doses of rMal d 1 (1.6, 3.13, 6.25 12.5, 25, 50 µg) as described previously²². Briefly, the challenge solution (75 µl of 0.9% w/v NaCl containing no or the indicated concentrations of rMal d 1) was pipetted directly under

the tongue and the participants were asked not to swallow for 2 minutes. The dose was increased every 20 minutes until relevant objective symptoms such as erythema, blister formation, edema and swelling of lips, tongue and larynx occurred up to a maximum dosage of 50 µg rMal d 1. None of the individuals reacted to 0.9% w/v NaCl alone.

Skin testing

Skin testing was performed on the flexor aspect of the forearms with a standardized prick needle (prick-test-lancets, ALK-ABELLO) with BPE (ALK-ABELLO), fresh apple (Pink Lady) and titrated concentrations (1.6-50 µg/ml) of rMal d 1 and rBet v 1 as described²³. Histamine and 0.9% w/v NaCl served as positive and negative control, respectively. Reactions were recorded after 20 minutes. The area of wheals was evaluated using ImageJ (Rasband W.S., National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2016).

Allergen-specific antibody responses

Serum levels of rBet v 1 and rMal d 1-specific IgE and IgG4 antibodies (Ab) were measured with ImmunoCAP 100 (Thermo Fisher Scientific). The IgG4/IgE ratio was calculated after conversion of kU_A/L of IgE to ng/ml by multiplication with 2.4.

Efficacy assessments

The primary end point of efficacy was the change from baseline to the end of treatment (16 weeks) of the dose of rMal d 1 inducing relevant objective symptoms in SCT. Secondary and exploratory outcomes included changes in skin responses and Ab responses to rMal d 1.

Statistical analysis

This pilot study was explorative in nature. For the proportion of AE in the three treatment groups at different time points we computed 95% Clopper Pearson confidence intervals. Longitudinal differences were tested with the Wilcoxon Signed Ranks test except when indicated differently. Differences between the three treatment groups were tested with rank based ANCOVA analysis using ranks of baseline measurements as covariates. For pairwise comparisons of the groups we applied Tukey PostHoc Tests. A P-value of <0.05 was considered statistically significant. Statistical analysis was performed using software SPSS10.01 and SAS9.4 (SAS Institute, Cary NC).

RESULTS

Subjects

Sixty participants with BP-associated apple allergy as determined by positive SCT with rMal d 1 were randomized into three groups of 20 individuals each to sublingually receive placebo, rMal d 1 (25 µg/day) or rBet v 1 (25 µg/day) for 16 weeks (Fig. 1). Table I shows participants' demographics and baseline characteristics for each group. All participants displayed IgE specific for BPE, rBet v 1 and rMal d 1. Two participants in the Bet v 1-group and two participants in the placebo group were negative for IgE to apple extract. One participant in the placebo group and one participant receiving Bet v 1 withdrew voluntarily because of adverse events (AE, see Table E2 in the Online Repository). Two participants in the Bet v 1-group withdrew voluntarily because of lack of time and moving away from Vienna, respectively. Taken together, 56 participants received the planned treatment (Fig. 1).

Safety analysis

No severe or systemic AE were recorded during the entire course of the study (see Table E2 in the Online Repository). All AE were considered to be treatment-emergent by the blinded investigator as the participants rated all symptoms including headache, eye edema, and conjunctivitis, as similar to symptoms when eating an apple. To obtain a detailed comparison of AE induced by rBet v 1 and rMal d 1 all symptoms including subjective reactions were recorded (see Table E2 in the Online Repository). After the first sublingual administration, 19 (95%) participants in each of the active groups and 11 (55%) participants in the placebo group reported at least one AE (see Table E2 in the Online Repository). To simplify the monitoring of the progression of AE during the course of the study we summarized oral and ear pruritus, oral blisters, edema, and/or throat irritation, as OAS. Figure 2 shows that at each time point the

percentages of participants reporting OAS were comparable between SLIT with rMal d 1 and rBet v 1 and much lower in the placebo group. Over time, the number of participants with OAS declined in all groups.

Efficacy analysis

Participants were sublingually challenged with increasing doses of rMal d 1 in December 2012 - January 2013 to exclude any possible effect of seasonal exposure to BP. SCT were repeated in the same time period after treatment (December 2013-January 2014). The doses of rMal d 1 inducing relevant oral symptoms at both time points were recorded for each individual. Overall, higher doses of rMal d 1 were needed after SLIT with rMal d 1 ($P<0.001$, Fig. 3). In detail, 14/20 rMal d 1-treated participants (70%) responded to higher challenge doses after treatment. Notably, 5/20 participants (25%) tolerated the highest dose of rMal d 1 and a subsequent challenge with 20 g of fresh apple. Two individuals (10%) developed OAS to lower doses and four individuals (20%) to the same dose of rMal d 1 after treatment. In contrast, no longitudinal differences in SCT were found in the 16 rBet v 1-treated participants (Fig. 3). In detail, 7/16 (44%) participants improved their apple allergy. One of these individuals (6%) tolerated all doses of rMal d 1 and fresh apple. 6/16 (38%) participants developed OAS to lower doses and 3/16 individuals (19%) to the same dose of rMal d 1 after treatment. In the placebo group 8/19 participants (42%) improved and five (26%) worsened in SCT. Six participants (32%) responded to the same dose of rMal d 1 before and after treatment.

A significant difference of rMal d 1 doses required to induce OAS after treatment was demonstrated between the three groups in Rank based ANCOVA analysis (F-Test, $P=0.0011$). The rMal d 1-treated group differed significantly from both the placebo

and the rBet v 1-treated group ($P=0.001$ and $P=0.038$, respectively, Tukey PostHoc Tests). The rBet v 1-treated group did not differ from the placebo group.

Skin test reactivity

In December 2012 - January 2013 all 60 participants underwent SPT with histamine, BPE, fresh apple, and titrated concentrations of recombinant allergens (see Figure E5 in the Online Repository). For comparison of skin reactivity of the 56 participants before and after treatment we choose the concentrations of 25 $\mu\text{g/ml}$ of rBet v 1 and 50 $\mu\text{g/ml}$ of rMal d 1 as they induced wheal areas comparable to commercial BPE at baseline (see Figure E5 in the Online Repository). SLIT with rMal d 1 led to a longitudinal decrease of wheal areas to rMal d 1 ($P=0.022$) whereas skin reactivity to fresh apple, rBet v 1, BPE, and histamine did not change (Fig. 4). SLIT with rBet v 1 significantly decreased skin reactivity to rBet v 1 ($P=0.023$) and BPE ($P=0.031$, Fig. 4) whereas skin reactivity to rMal d 1 and fresh apple remained unchanged. In the placebo group no changes of wheal areas to any of the tested agents were observed.

Immune responses

IgE and IgG4 levels specific for rBet v 1 and rMal d 1 were measured in sera collected at baseline and after treatment. SLIT with rMal d 1 induced rMal d 1-specific IgG4 Ab ($P<0.001$) but neither affected rBet v 1-specific IgE and IgG4 nor rMal d 1-specific IgE levels (Fig. 5). In contrast, SLIT with rBet v 1 induced IgE and IgG4 Ab specific for both allergens. Except for Mal d 1-specific IgG4 these levels were significantly increased compared to those in participants who received rMal d 1 and placebo (Fig. 5). In the placebo group no remarkable changes of allergen-specific Ab levels before and after treatment were observed. The calculation of rMal d 1-specific IgG4/IgE ratios revealed a longitudinal increase in participants treated with rMal d 1

277 (P=0.012), a decrease in participants treated with rBet v 1 (P=0.049) and no change
278 in the placebo group (Fig. 5).

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DISCUSSION

One novelty of this pilot study is the investigation of the safety, efficacy and immune effects of a sublingually administered recombinant food allergen. A second innovation is the use of defined amounts of rMal d 1 for standardized assessment of food-induced clinical responses before and after treatment. Former studies have relied on prick-to-prick tests and/or food challenges with fresh apples. However, these test methods cannot guarantee the use of constant amounts of Mal d 1 for challenges at different time points as its content in apples strongly depends on the cultivar, stage of maturation and storage conditions^{24, 25}. Moreover, Mal d 1 is easily destroyed during the preparation of the test meals because this protein is extremely susceptible to acidic conditions and proteases²⁶. Another important feature of this trial is the parallel analysis of all parameters induced by equal doses of rMal d 1 and rBet v 1 which for the first time allow the direct comparison of SLIT with two homologous allergens, displaying 56% of sequence identity (71% homology) and a common three-dimensional fold²⁷.

Daily sublingual administration of 25 µg of rMal d 1 for 16 weeks was safe and induced an improvement of apple allergy compared to placebo. Notably, 70% of the participants showed an increased rMal d 1-threshold in SCT and 25% of the participants tolerated a cumulative amount of 98.8 µg of rMal d 1 plus 20 g of fresh apple after treatment. After 44 weeks of peanut SLIT, 70% of 20 subjects receiving peanut SLIT were responders compared with 15% of 20 subjects receiving placebo²⁸.

A commercialized SLIT with peach extract for 12 months enhanced the threshold of peach in food challenges in 92% of 23 individuals²⁹. This clinical improvement was accompanied by a reduction of skin reactivity and enhanced Pru p 3-specific IgG4/IgE ratios²⁹. SLIT with rMal d 1 also significantly reduced the skin reactivity to rMal d 1 but not to rBet v 1 and a significant rise of rMal d 1-specific IgG4/IgE ratios.

Transient tolerance to apple in patients with birch pollen-related apple-allergy has been achieved by oral immunotherapy (OIT), i.e. daily intake of increasing amounts of fresh apple for an average time interval of 20 weeks²⁰. However, this desensitization with unknown doses of the major apple allergen was neither accompanied by improved skin reactivity nor enhanced allergen-specific IgG4 levels and referred to the treatment-induced exhaustion of local mast cells²⁰. We observed a clear alteration of the rMal d 1-specific immune response indicative of the induction of immune tolerance³⁰. In addition, two participants in the rMal d 1-group reported to be still tolerant to apple two years after the treatment (data not shown). We conclude that giving a constant sublingual dose of the major apple allergen is necessary to effectively treat birch pollen-associated apple allergy.

This conclusion is further supported by the notably different clinical and immunological effects of SLIT with 25 µg of rBet v 1. Overall, these participants did not improve their sublingual reactivity to rMal d 1 although they experienced a similar extent of treatment-emerged OAS during the course of treatment (Fig. 2). Thus, despite the exhaustion of sublingual mast cells by rBet v 1 no desensitization to the highly cross-reactive rMal d 1 was achieved. This limited improvement of the associated apple allergy matches our previous results of a yearlong SLIT with birch pollen extract demonstrating that apple allergy improved in 33%, worsened in 33% and remained unaffected in 33% of the participants with improved birch pollinosis¹⁹. In another trial, birch pollen SLIT improved apple allergy in only 1/11 individuals but induced food allergy in 3/11 individuals¹⁶. One of our rBet v 1-treated participants also tolerated the full SCT procedure with rMal d 1 and 20 g of fresh apple indicating that a limited number of individuals may benefit from SLIT with the pollen allergen. Recently, daily sublingual administration of 25 µg of rBet v 1 in fast dissolving tablets for 5 months has been reported to effectively improve birch pollinosis³¹. Our

participants displayed significantly reduced skin reactivity to rBet v 1 and BPE as well as enhanced rBet v 1-specific IgE and IgG4 Ab responses. In parallel, rMal d 1-specific IgE and IgG4 levels significantly rose during treatment. We assign this finding to the induction of cross-reactive rBet v 1-specific Ab. However, the ratio of rMal d 1-specific IgG4/IgE Ab decreased during treatment. In summary, we demonstrate that equal doses of two homologous and highly cross-reactive allergens administered directly at the site of food-induced allergic symptoms induce surprisingly different clinical and immunological responses. Notably, SLIT with rBet v 1 showed limited clinical efficacy on BPRFA. As allergic reactions to apple are one of the best studied examples of BPRFA, we conclude that BPRFA cannot be treated effectively with the primary sensitizer, i.e. the major birch pollen allergen.

We have previously demonstrated that rMal d 1 improves the sensitivity and specificity of *in vitro* and *in vivo* diagnosis of BPRFA in 21 BP-allergic patients^{22, 23}. This trial substantiates these findings in additional 60 BP-allergic individuals and additionally illustrates that the recombinant apple protein is very practical for SLIT. The vast majority of subjects considered the study drug to be easy to handle and neutral in taste (see Table E2 in the Online Repository). In contrast to its native and extremely labile counterpart, the recombinant major apple allergen proved to be biochemically and immunologically stable which seems relevant for the success of treatment (see Table E1 and Figures E1-4 in the Online Repository). In view of the relatively short treatment duration of 16 weeks and the small study population, the observed clinical and immunological effects of SLIT with rMal d 1 are significant. In summary, daily sublingual administration of rMal d 1 is a promising approach for safe and effective treatment of birch pollen-associated apple allergy.

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460 **Table I.** Demographics and baseline characteristics of the participants

Characteristics	Bet v 1 (n=17)	Mal d 1 (n=20)	Placebo (n=19)
Sex (female/male)	13/7	7/13	11/9
Age (y) median (range)	38 (20-64)	32.5 (21-64)	33 (21-53)
BP-specific IgE (kU_A/L)			
Median	11.4	20.8	17.2
range	1.4-38.3	2.0-59.9	4.5-54.3
Bet v 1-specific IgE (kU_A/L)			
Median	11.4	20.5	13.6
range	1.5-31.7	3.8-45.9	4.3-57.6
Apple-specific IgE (kU_A/L)			
Median	1.9	1.7	2.1
range	<0.35-8.4	0.4-45.7	<0.35-1.9
Mal d 1-specific IgE (kU_A/L)			
Median	5.1	7.2	6.4
range	0.6-14.8	0.9-45.2	2.2-24.1

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FIGURE LEGENDS

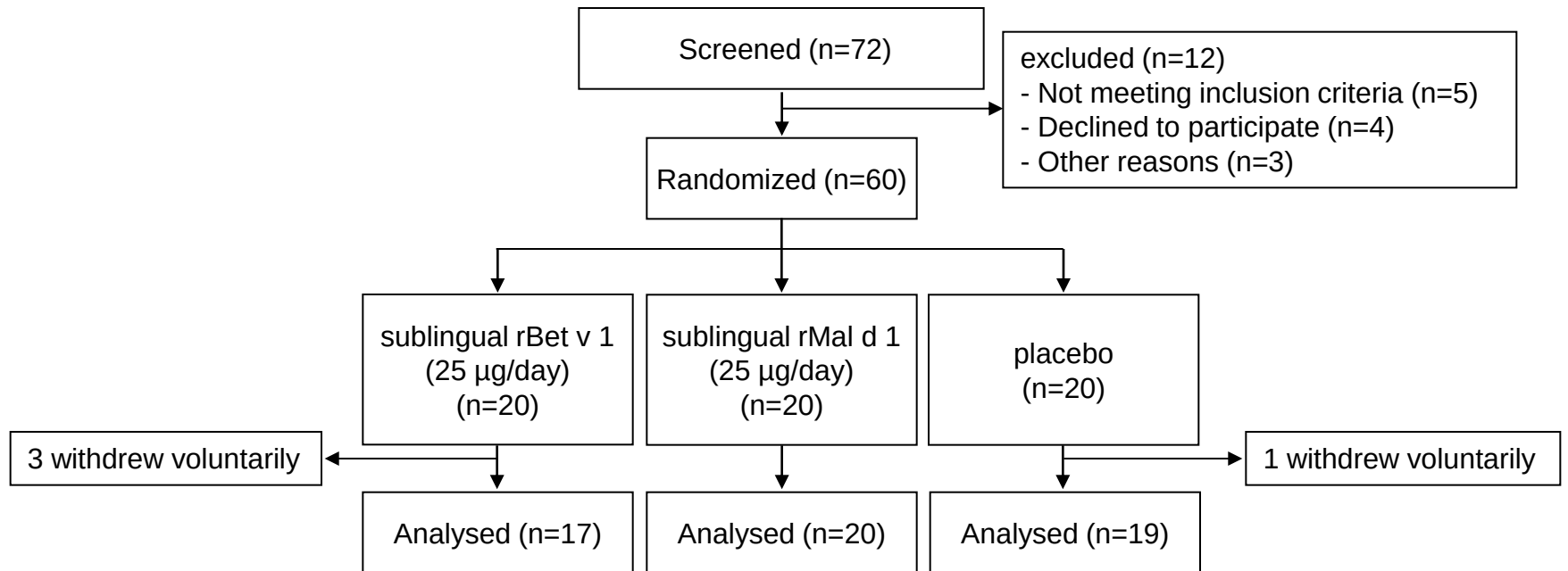
Figure 1. Trial profile. Flow diagram illustrating the numbers of participants screened, randomized, treated and analysed during the study.

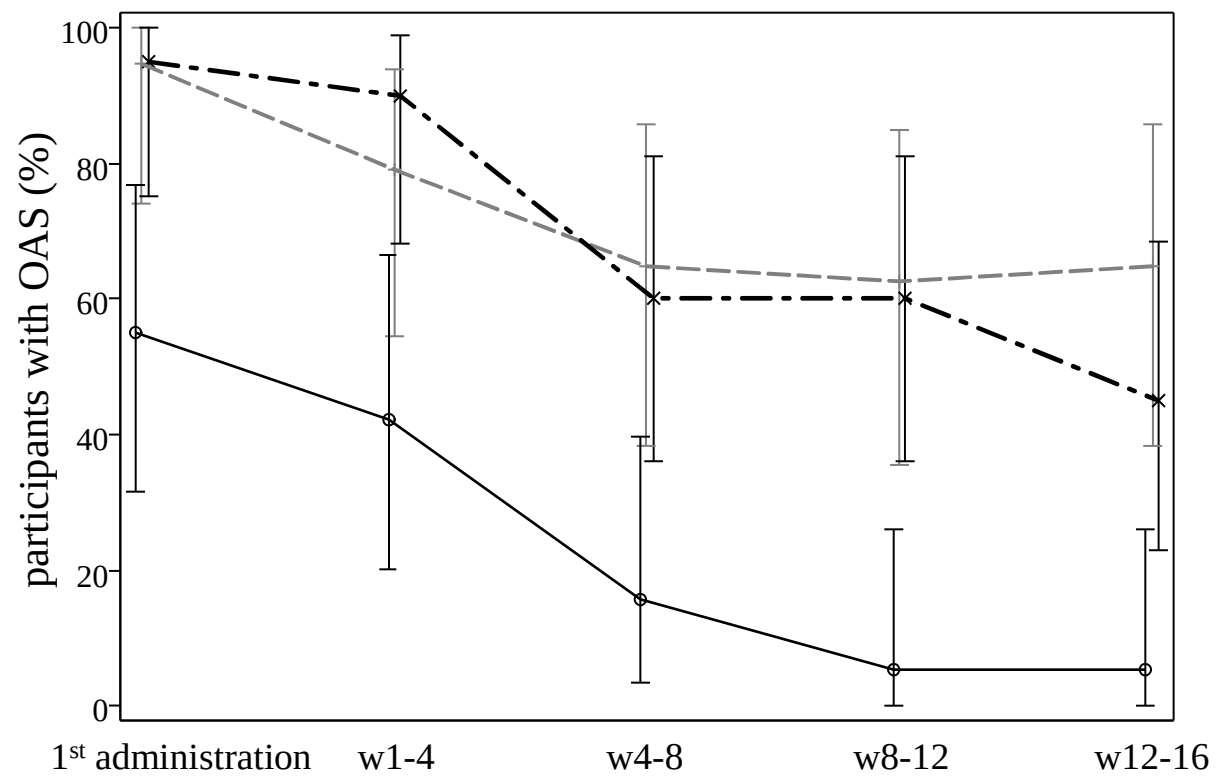
Figure 2. Monitoring of adverse events. Adverse events were recorded after the first dose of placebo (black line), rBet v 1 (gray dashed line) and rMal d 1 (black dashed line) and in four-weekly intervals during the treatment period. Nasopharyngeal reactions (see Table E2 in the Online Repository) were summarized as OAS. The percentages of participants with OAS together with 95% Clopper Pearson confidence intervals are shown; w, week;

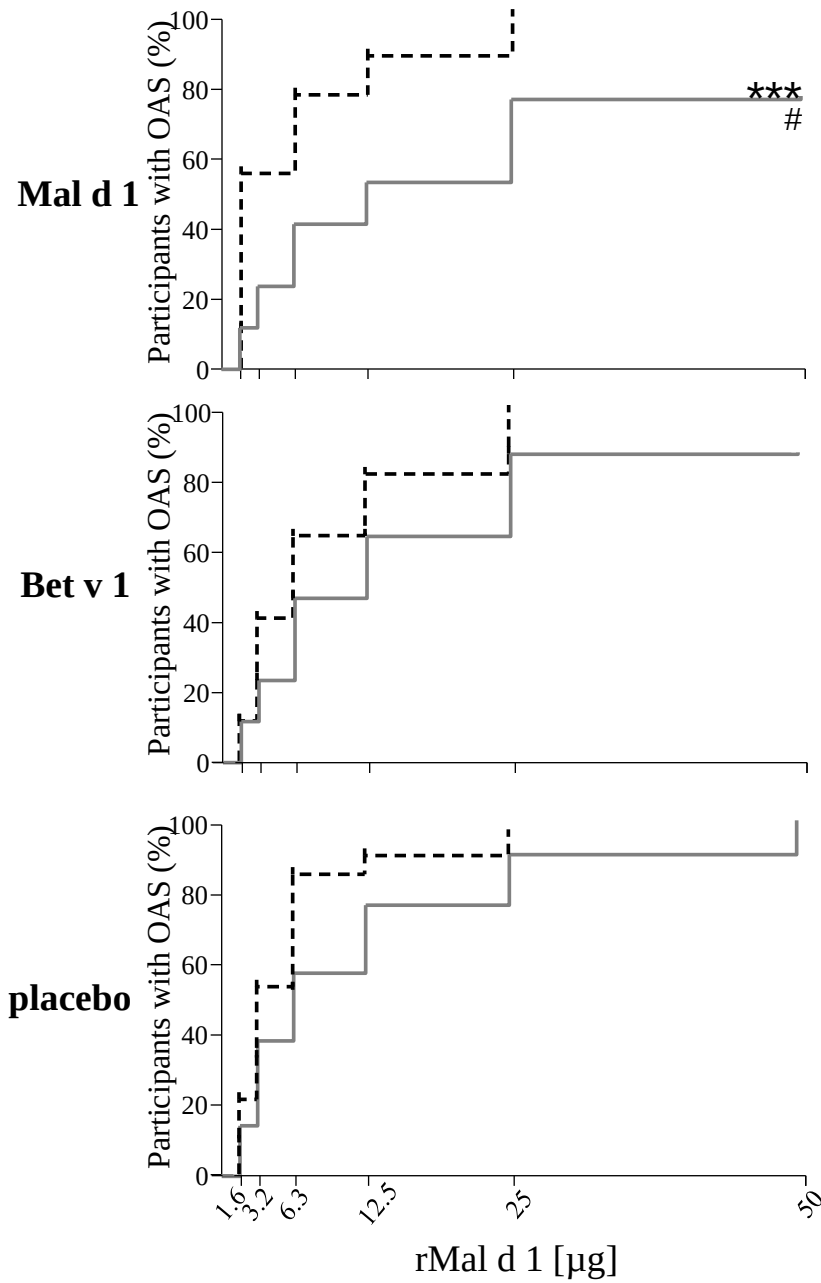
Figure 3. Treatment efficacy. Sublingual challenge tests performed with increasing doses of rMal d 1 until OAS occurred before (black dashed line) and after 16 weeks (gray line) of SLIT with rMal d 1, rBet v 1 and placebo, *** $P < 0.001$, Wilcoxon Signed Ranks test. #After treatment, the Mal d 1-treated group differed from the Bet v 1-treated ($P = 0.038$) and the placebo group ($P = 0.001$), Tukey PostHoc Tests in Rank based ANCOVA.

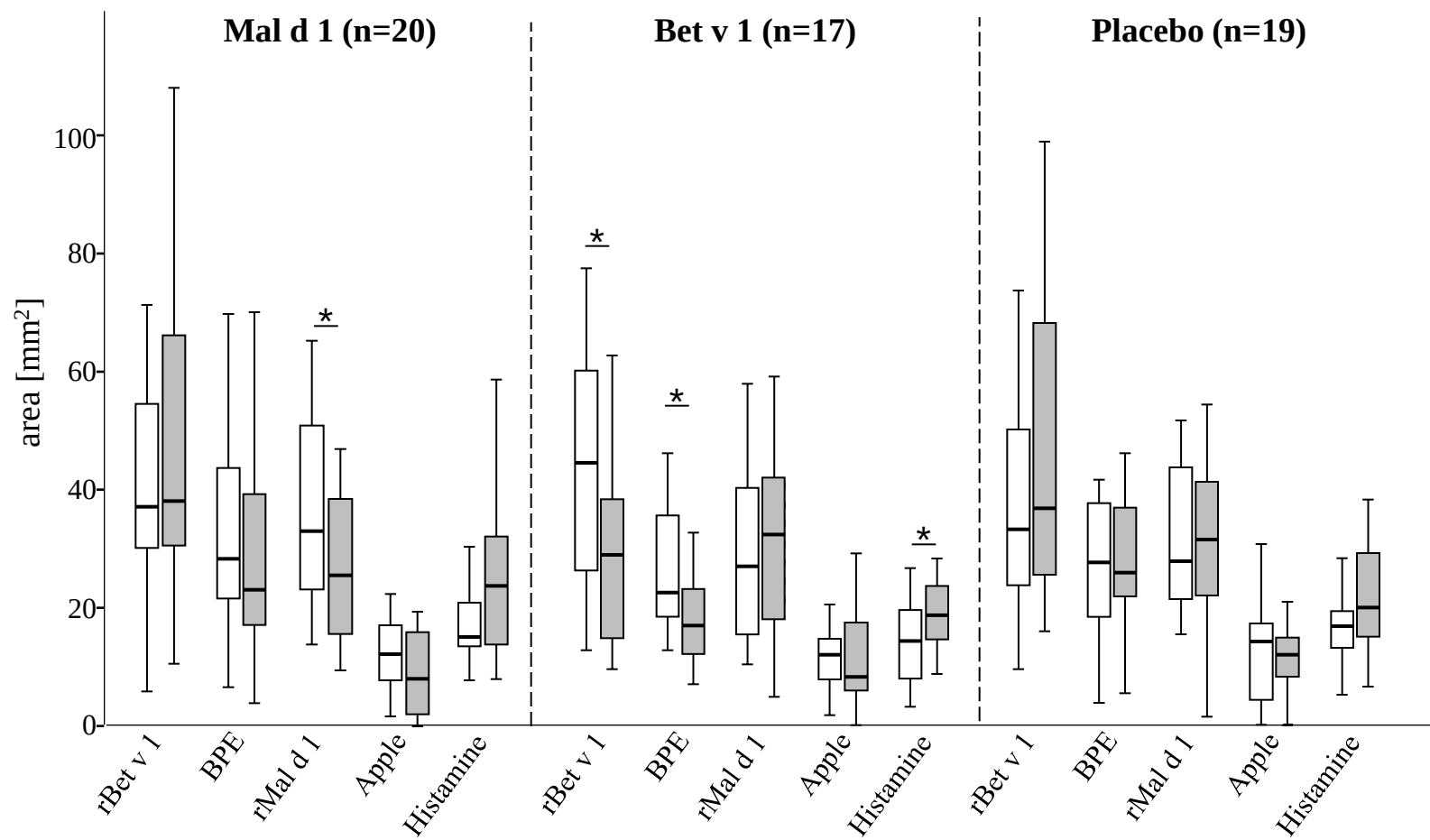
Figure 4. Effect of treatment on skin reactivity. Skin testing with rBet v 1, rMal d 1, birch pollen extract (BPE), fresh apple (Pink lady) and histamine was performed at baseline (white box plots) and after 16 weeks of treatment (gray box plots). The area of wheals was determined by ImageJ, * $P < 0.05$, Wilcoxon Signed Ranks test.

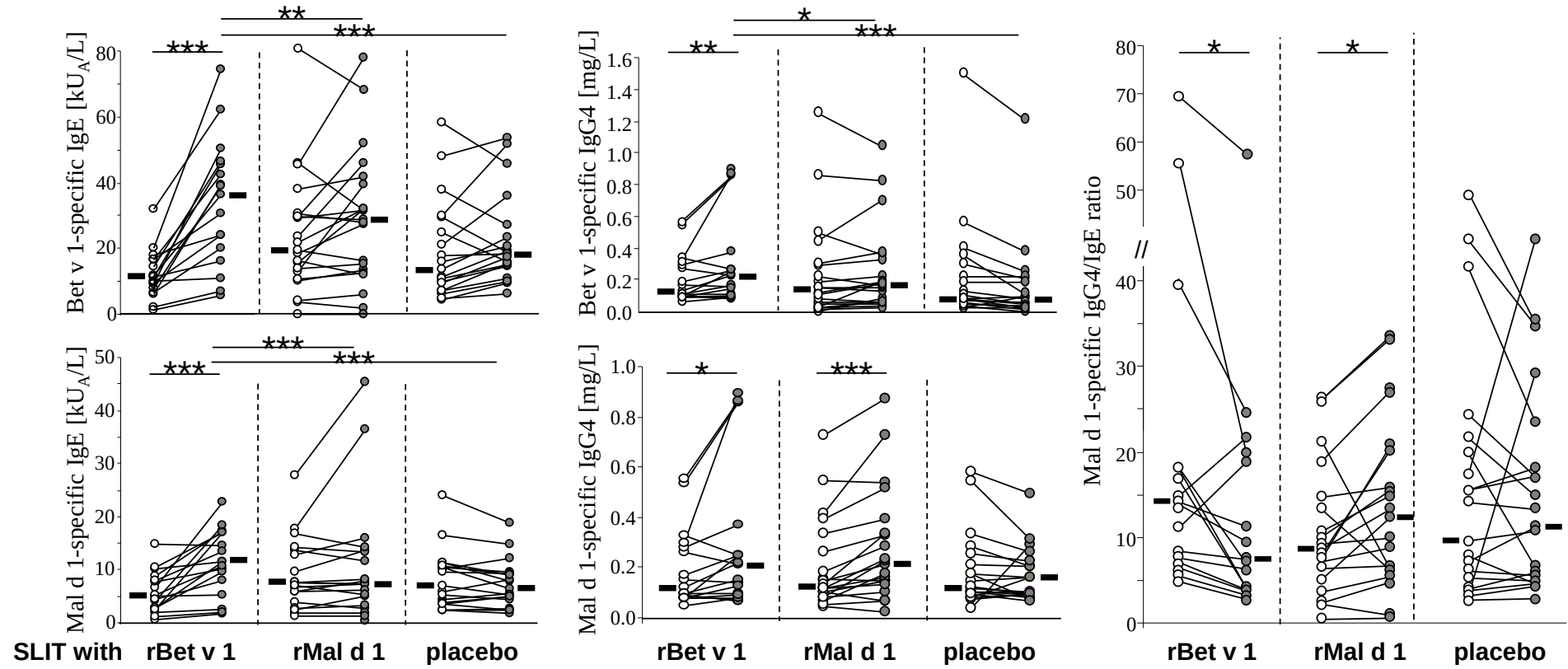
Figure 5. Effect of treatment on allergen-specific antibody levels. Bet v 1- and Mal d 1 specific IgE and IgG4 levels at baseline (white dots) and after 16 weeks of SLIT (grey dots) with either rBet v 1, rMal d 1 or placebo. Mal d 1-specific IgG4/IgE ratios were calculated after conversion of kU_A/L of IgE to mg/ml. Lines indicate median values, *P<0.05, **P<0.01, ***P<0.001, Wilcoxon Signed Ranks test.











ONLINE REPOSITORY**Efficacy and safety of 4 months of sublingual immunotherapy with recombinant Mal d 1 in patients with birch pollen-related apple allergy**

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Characterization of the investigational products

Bet v 1.0101-GMP-0802 and Mal d 1.0108-GMP-0902 were produced under GMP conditions by Biomay AG, Vienna, Austria. For sublingual application Bet v 1.0101-GMP-0802 was dissolved in 5 mM phosphate buffer (pH=7.4) and Mal d 1.0108-GMP-0902 in 20 mM carbonate buffer (pH=9.0), supplemented with 25% v/v glycerol and kept in dark glass flasks at 4°C. These solutions were termed KLI96rBet v 1 and KLI96rMal d 1, respectively. The concentration of each allergen was regularly monitored by using the bicinchoninic acid assay (BCA, Sigma Aldrich, Vienna, Austria) according to the manufacturers' instructions (Table E1). The integrity of both allergens under these conditions after 6 months of storage at 4°C was tested by means of SDS-PAGE (Fig. E1), immunoblotting (Fig. E2), ELISA (Fig. E3), and HP-SEC (Fig. E4) and compared to reference allergens, i.e. rBet v 1.0101 and rMal d 1.0108 stored under optimum conditions for each allergen.

Table E1. Concentration [$\mu\text{g/mL}$] of KLI96rBet v 1 and KLI96rMal d 1 at 4°C

	Months				
	0	1	2	3	6
KLI96rBet v 1	92*	88	91	80	90
KLI96rMal d 1	93	88	83	89	84

*Due to the variability of the BCA, values in the range of 71.4-96.6 $\mu\text{g/ml}$ are considered acceptable.

Figure E1. Integrity of KLI96rBet v 1 and KLI96rMal d 1 as determined by SDS-PAGE

Reference allergens (1 µg in lanes 1 and I) and different concentrations of KLI96rBet v 1 and KLI96rMal d 1 (1, 0.75, 0.50, 0.25 and 0.1 µg per lane 2-6 and II-VI, respectively) after storage for 6 months at 4°C were separated using TRIS-HCl gels (10-20%, Bio-Rad Criterion, Bio-Rad, Vienna, Austria) under reducing (1-6) and non-reducing (I-VI) conditions and visualized by Coomassie Brilliant blue staining. M, marker proteins (Bio-Rad)

Figure E2. Antibody-binding activity of KLI96rBet v 1 and KLI96rMal d 1 as determined by immunoblotting

Allergens were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Membranes were incubated with a monoclonal mouse anti-Bet v 1 antibody (lanes 1 and 2) and anti-Bet v1aF1 IgG (lanes 3 and 4). Bound antibodies were detected with an alkaline phosphatase-conjugated anti-mouse IgG antibody (Sigma Aldrich). M, marker proteins (Bio-Rad); lanes 1 and 3, reference allergens; lanes 2 and 4, KLI96rBet v 1 and KLI96rMal d 1 after storage for 6 months at 4°C; lanes 5 and 6, buffer controls;

Figure E3. IgE-binding activity of KLI96rBet v 1 and KLI96rMal d 1 as determined by ELISA

Microtiter plates (Maxisorp, Nunc, Denmark) were coated with the indicated concentrations of reference allergens (grey diamonds), KLI96rBet v 1 and KLI96rMal d 1 after storage for 6 months at 4°C (black triangles), respectively, in carbonate buffer (pH=9.6) overnight at room temperature. After saturation with 1% w/v BSA in PBS for 60 minutes, a serum pool consisting of four allergic patients was incubated

for 2 hours at room temperature. After washing, bound IgE was detected using an alkaline phosphatase-conjugated anti-human IgE antibody (BD Biosciences Pharmingen, San Diego, CA). O.D. optical density

Stored rBet v 1 showed a slightly reduced IgE-binding ability, however, the deviation at the concentrations of 1 and 10 µg/ml, respectively, was not significantly different from the reference allergen.

Figure E4. Characterization of KLI96rBet v 1 and KLI96rMal d 1 by high performance size exclusion chromatography (HP-SEC)

Reference rBet v 1 and rMal d 1 (A, C) and KLI96rBet v 1 and KLI96rMal d 1 stored for 6 months at 4°C (B, D) were loaded (8.4 µg each) onto a Phenomenex Poly-SEP-GFC-P4000 column with a void volume of 4.3 ml. Monomeric Bet v 1 appeared at a retention time of approx. 45.3 min in both samples (A and B). Dimeric Mal d 1 appeared at a retention time of approx. 44.1 min followed by monomeric Mal d 1 at a retention time of 45.3 min (B, D). The proportions of monomeric (88-90%) and dimeric Mal d 1 (10-12%) was similar in both samples. The positive and negative peaks result from the 25% w/w glycerol in the buffer.

95 **Table E2.** Adverse events (AE) reported by patients after the first application of the investigational products and at four-weekly
 96 intervals during the treatment period

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Patient No	Treatment	AE after first dose	AE during week 1-4	AE during week 4-8	AE during week 8-12	AE during week 12-16
1	Mal d 1	mild oral pruritus for 20 min	moderate oral pruritus for 10 min	very mild oral pruritus for 1-2 min	mild oral pruritus for 2 min	very mild oral pruritus for 2 min
2	Bet v 1	itching at the application site for 10 min	Local papules and blisters, local itching and redness of eyes	no complaints	no complaints	no complaints
3	Bet v 1	oral pruritus and small blisters on lips for 40 min	oral pruritus for 5 min	Mild oral pruritus and sublingual blisters and on the lips for 5-10 min	Mild oral pruritus and blisters sublingual and on the lips	Mild oral pruritus for 3-5 min
4	Mal d 1	ear pruritus and throat irritation for 30 min	mild to moderate throat irritation only in the first week	no complaints	Occasional throat irritation after swallowing	no complaints
5	Bet v 1	Moderate oral pruritus for 20 min	Tingling at the application site for 2-5min	Mild tingling at the application site	Mild tingling at the application site	Mild tingling at the application site
6	Bet v 1	Moderate throat irritation for 45 min	Mild itching at the application site for 10 min	no complaints	no complaints	no complaints
7	Mal d 1	Mild oral and ear pruritus for 40 min after application	Mild to moderate itching of the eyes for 15 min	Mild itching in the throat, ears and eyes for 15 min	oral pruritus and mild erythema of the left eye Much milder then at the beginning	Mild oral pruritus
8	Mal d 1	Moderate furry feeling and pettiness on tongue, throat and cheek mucosa for 15 min.	Erythema of the eyes in the 1. Week, itching at the application site in the 2. Week, now mild itching on the tongue tip	no complaints in the mouth but sometimes running nose for 20 min	no complaints	no complaints
9	Bet v 1	oral pruritus and throat irritation Difficulties in swallowing	Mild pettiness in the mouth for 3-4 min. after application	no complaints	no complaints	no complaints

		for 30 min. - similar to the symptoms when eating an apple				
10	Placebo	no complaints	no complaints	no complaints	no complaints	no complaints
11	Placebo	Cough for 5-6 min	Withdrew voluntarily	---	--	--
12	Mal d 1	Oral pruritus, throat irritation, mild swelling of the lips for 10 min	Mild itching at the application site	no complaints	Sometimes mild tingling for 5 min.	no complaints
13	Bet v 1	Moderate oral pruritus for 10 min	Mild to moderate oral pruritus for 5-7 min	Mild oral pruritus for 5 min	Very mild oral pruritus for 3 min.	Very mild sublingual itching for 1 min.
14	Bet v 1	Mild oral pruritus	Moderate oral pruritus for 10 min	Moderate oral pruritus for 5min	Burning sensation on the palate and tongue tip for 2- min	Mild throat irritation for 1 min
15	Bet v 1	Moderate oral pruritus for 5 min	Mild oral pruritus and throat irritation	Withdrew voluntarily	---	---
16	Mal d 1	Mild oral and ear pruritus	In the first week cough, ear pruritus, throat irritation Last week no complaints	no complaints	no complaints	no complaints
17	Placebo	no complaints	no complaints	no complaints	no complaints	no complaints
18	Placebo	no complaints	no complaints	no complaints	no complaints	no complaints
19	Placebo	Mild itchy eyes for 1 h	no complaints	no complaints	no complaints	no complaints
20	Placebo	Mild oral pruritus for 40 min	no complaints	no complaints	no complaints	no complaints
21	Mal d 1	Itching burning in the mouth for 10 min	no complaints	no complaints	no complaints	Mild itching on the palate for 2-3 min
22	Mal d 1	oral pruritus and throat irritation for 20 min	Mild oral pruritus and throat irritation	Mild oral pruritus and throat irritation for 5 min	no complaints	Mild sublingual itching for 3-4 min.
23	Placebo	no complaints	Mild sublingual tingling for 2 min	no complaints	no complaints	no complaints
24	Placebo	oral and ear pruritus and throat irritation for 20 min.	The first 3 days mild sublingual itching, thereafter no complaints	no complaints	no complaints	no complaints
25	Bet v 1	Mild oral pruritus for 10 min	no complaints	Mild oral pruritus for 2 min	no complaints	Mild oral pruritus 2 min in the last 3 days
26	Mal d 1	Moderate oral pruritus for 10 min	Occasional moderate itching of lips and	Mild scratching on the palate	no complaints	no complaints

			tongue			
27	Placebo	no complaints	Occasional mild itching of lips	no complaints	no complaints	no complaints
28	Mal d 1	Moderate sublingual itching for 30 min	Moderate burning and itching of the lower lip for 15 min	Occasional mild burning and itching of the lower lip for 5-10 min	Occasional mild burning and itching of the lower lip	Occasional mild burning and itching of the lower lip
29	Placebo	Very mild oral pruritus for 15 min	The first 2 weeks mild sublingual itching, thereafter no complaints	no complaints	no complaints	no complaints
30	Mal d 1	Moderate throat irritation for 45 min	Moderate pettiness in the mouth, throat and on the tongue for 30-45 min	Mild tingling	mild throat irritation for 10 min	no complaints
31	Mal d 1	Moderate oral pruritus and throat irritation for 40 min	Mild oral pruritus and throat irritation during the past 14 days running nose	no complaints	Occasional oral pruritus	no complaints
32	Mal d 1	Pettiness of the tongue, mouth and throat, Warmth in the chest for 90 min	itching of the tongue in the first 2 weeks, thereafter no complaints	Sometimes mild sublingual pettiness for 5-10 min	Mild tingling sublingual	no complaints
33	Bet v 1	Mild to moderate oral and ear pruritus and throat irritation for 40 min	no complaints	Mild itching on the palate and in the ears for 10 min	Mild throat irritation	Mild pettiness of the lower lip
34	Placebo	no complaints	Mild oral pruritus for 1 min	Mild tingling only on the tongue tip for 1 min	Occasional mild sublingual tingling	Occasional mild sublingual tingling
35	Mal d 1	Moderate oral pruritus for 5 min	no complaints	no complaints	no complaints	no complaints
36	Mal d 1	Throat irritation for 10 min	Mild oral pruritus for 2 min	Occasional mild throat irritation for 2 min	Occasional mild throat irritation for 2 min	Occasional mild throat irritation for 2 min
37	Mal d 1	Oral pruritus for 30 min	Occasional mild oral pruritus Sometimes for 3-5 Min	no complaints except during 4 days of a cold with moderate oral pruritus for 30 min	Occasional mild oral pruritus Sometimes for 2-3 min	no complaints
38	Bet v 1	Oral pruritus and throat	Occasional mild tingling	no complaints	Occasional mild tingling	Occasional mild tingling

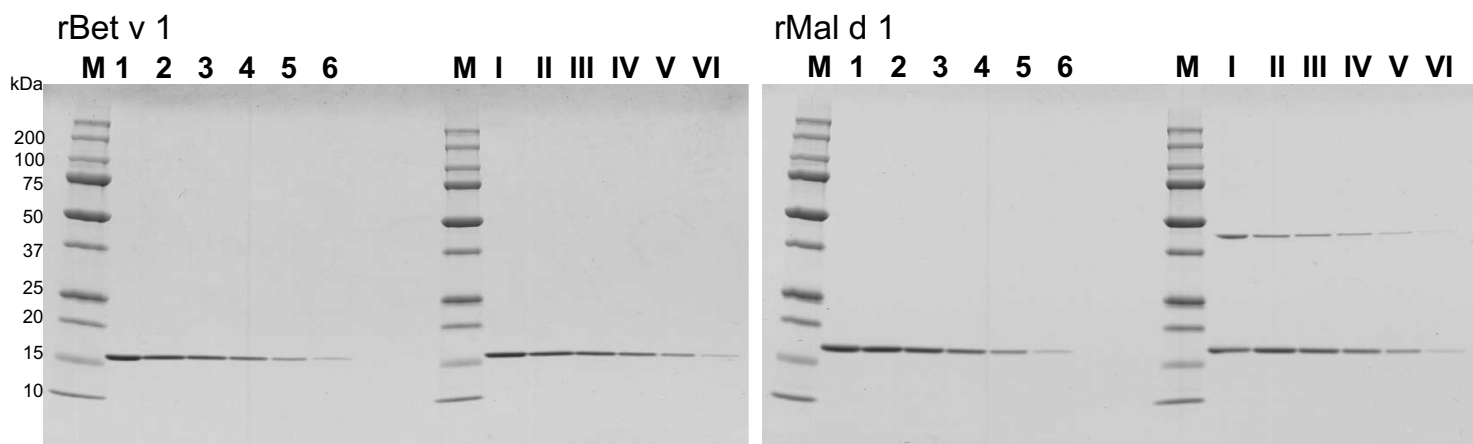
		irritation for 10 min	of the tongue for 5-10 min		of the tongue for 5 min	of the tongue for 5 min
39	Bet v 1	Oral and ear pruritus for 30 min	Moderate to severe oral and ear pruritus for 1-4 h	Moderate to severe oral and ear pruritus for 1-4 h	Moderate to severe oral and ear pruritus for 1-3 h	Moderate to severe oral and ear pruritus for 1-3 h
40	Bet v 1	no complaints	Sublingual mild- moderate tingling for 10-15 min	Mild throat irritation after swallowing for 2 min	no complaints	no complaints
41	Placebo	Mild sublingual pettiness for 40 min	no complaints	no complaints	no complaints	no complaints
42	Bet v 1	withdrew voluntarily	----	----	----	----
43	Bet v 1	Moderate oral pruritus and throat irritation for 30 min	Mild oral pruritus	no complaints	Mild itching after swallowing of the medication for 5 min	Occasional mild tingling of lower lip for 5 min
44	Bet v 1	Moderate oral pruritus and throat irritation	mild tingling of the tongue tip	Moved from Vienna and withdrew voluntarily	----	----
45	Placebo	no complaints	no complaints	no complaints	no complaints	no complaints
46	Mal d 1	Mild itching on the tongue tip for 25 min	Mild itching on the tongue tip and ear pruritus for 1-2 min	no complaints	no complaints	Only once mild ear pruritus
47	Bet v 1	Moderate oral pruritus and throat irritation for 20 min	no complaints	oral pruritus and throat irritation for 7-10 min	oral pruritus and throat irritation for 3-5 min	oral pruritus for 2 min
48	Bet v 1	Oral pruritus and throat irritation for 30 min	Moderate oral pruritus and throat irritation for 10 min, Increased production of saliva	Mild oral pruritus and throat irritation	Very oral pruritus Increased production of saliva	no complaints
49	Placebo	Mild sublingual tingling for 10 min	no complaints	no complaints	no complaints	no complaints
50	Placebo	no complaints	no complaints	no complaints	no complaints	no complaints
51	Placebo	no complaints	no complaints	no complaints	no complaints	no complaints
52	Placebo	Sublingual tingling for 10 min	no complaints	Occasional very mild sublingual tingling for 2 min	no complaints	no complaints
53	Mal d 1	no complaints	Very mild itching on the tongue tip for 1 min	very mild tingling	no complaints	no complaints

54	Placebo	Headache for about 1 h, similar to the reaction when eating an apple	During the first week sublingual tingling for 5 min, thereafter no complaints	Occasional mild sublingual tingling for 2 min	no complaints	no complaints
55	Bet v 1	Mild tingling on the palate and tongue for 15 min	Mild sublingual tingling for 5-10 min	Occasional mild scratching in the throat	no control as the patient was abroad	Mild tingling on the tongue tip for 3 min
56	Placebo	Mild itching in the nose for 20 min	During the first 5 days of treatment mild tingling in fingers, thereafter no further complaints	no complaints	no complaints	no complaints
57	Mal d 1	Mild to moderate oral and ear pruritus	Mild itching on the palate	Mild itching on the palate	Mild itching on the palate	Mild itching on the palate for 2 min
58	Bet v 1	Mild tingling on the palate, in the throat for a few min	no complaints	no complaints	no complaints	no complaints
59	Placebo	Ear pruritus for 40 min	Mild oral pruritus and throat irritation	no complaints	no complaints	no complaints
60	Mal d 1	Mild sublingual tingling for 25 min	Mild sublingual tingling for 15 min	Mild sublingual tingling for 15 min	Mild sublingual tingling	Very mild tingling on the palate for 5-10 min

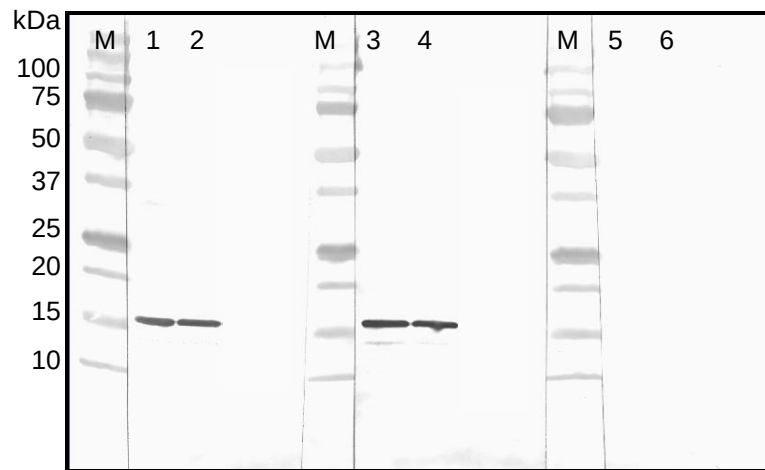
98 min, minutes; h, hours

Figure E5. Skin reactivity to titrated concentrations of rBet v 1 and rMal d 1

SPT was performed in 60 birch pollen-allergic patients in December 2012-January 2013 with titrated concentrations (1=50, 2=25, 3=12.5, 4=6.3, 5=3.2 and 6=1.6 µg/ml 0.9% w/v NaCl) of Bet v 1.0101-GMP-0802 and Mal d 1.0108-GMP-0902, commercially available birch pollen extract (BPE, ALK-ABELLO, Hørsholm, Denmark), fresh apple (Pink lady) and histamine. The area of wheals was determined by ImageJ. (**P<0.001, *P<0.05, Wilcoxon Signed Ranks test)



rBet v 1



rMal d 1

