

7. Zhang X, Simmerman K, Yen-Lieberman B, Daly TM. Impact of analytical variability on clinical interpretation of multiplex pneumococcal serology assays. *Clin Vaccine Immunol* 2013;20:957-61.
8. Daly TM, Pickering JW, Zhang X, Prince HE, Hill HR. Multilaboratory assessment of threshold versus fold-change algorithms for minimizing analytical variability in multiplexed pneumococcal IgG measurements. *Clin Vaccine Immunol* 2014;21:982-8.
9. Hajjar J, Al-Kaabi A, Kutac C, Dunn J, Shearer WT, Orange JS. Questioning the accuracy of currently available pneumococcal antibody testing. *J Allergy Clin Immunol* 2018;142:1358-60.

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Vaccination of nonallergic individuals with recombinant hypoallergenic fragments of birch pollen allergen Bet v 1: Safety, effects, and mechanisms



To the Editor:

Studies suggested that only allergen-specific immunotherapy (AIT) but not pharmacotherapy can prevent the progression of allergic rhinitis to asthma in children.¹ Recently conducted extensive analyses of the evolution of allergic sensitization in birth cohorts with microarrayed allergens demonstrate that silent IgE sensitization often precedes allergic symptoms and that the development of allergic symptoms may be predicted on the basis of IgE recognition of certain allergens already early in life.² In this context, not only AIT based on allergen extracts but also molecular AIT strategies have been considered for preventing the development of allergy in IgE-sensitized but not yet symptomatic children.^{3,4} As a first step toward prophylactic allergy vaccination based on a molecular AIT approach we have conducted a double-blind, placebo-controlled clinical trial, in which nonallergic subjects were vaccinated with recombinant hypoallergenic derivatives of the major birch pollen allergen, Bet v 1.^{5,6} We screened 44 subjects for the study of whom 16 were randomized, 10 to the placebo group and 6 to the actively treated group (see Fig E1 in this article's Online Repository at www.jacionline.org). Subjects in the active and placebo groups were similar regarding age and demographic parameters (see Table E1 in this article's Online Repository at www.jacionline.org). They lacked detectable IgE reactivity to birch pollen, mugwort pollen and grass pollen extract, and cat and house dust mite extract and were negative in skin prick tests with grass pollen, birch pollen, ash pollen, ragweed pollen, mugwort pollen, house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), molds (*Alternaria alternata* and *Cladosporium herbarum*), and cat and dog dander extract (Table E1). During the 2-year study, 3 subcutaneous injections of Alum-adsorbed rBet v 1 fragments or Alum alone (placebo) were administered in monthly intervals before the birch pollen season of the first year (ie, 2013) and a single booster injection was given before the birch pollen season of the second year (ie, 2014). The study design thus allowed monitoring the effects of vaccination and of natural birch pollen exposure on specific immune responses (Fig 1, A-F). The development of birch pollen-specific antibody, cellular and cytokine responses as well as skin sensitivity was repeatedly recorded throughout the 2 years as indicated in Fig 1, A. Fig E2 in this article's Online Repository at www.jacionline.org shows the development of IgG responses

toward Bet v 1 and the 2 Bet v 1 fragments, F1 and F2, for each of subjects in the active (Fig E2, A) and placebo groups (Fig E2, B) during the 2 years of the study. The quantitative measurement of Bet v 1-specific IgG₁ and IgG₄ subclass levels is shown in Fig E3, A and B, in this article's Online Repository at www.jacionline.org. Subjects from the active group as well as the placebo group showed preexisting IgG responses to Bet v 1 and Bet v 1 fragments already before the first vaccination. Most of the subjects vaccinated with the rBet v 1 fragments developed IgG against Bet v 1 and both fragments after the second injection and a significant increase in Bet v 1-specific IgG was found in all 6 actively treated subjects after the third injection (Fig E2, A; see Fig E4 in this article's Online Repository at www.jacionline.org). Thus, the primary end point of the study was reached (Online Repository: ClinicalTrials.gov Identifier: NCT01353924). No significant increases in Bet v 1-specific IgG levels were found in the placebo group at the same time point (ie, visit 6) (Fig E4). Natural exposure to birch pollen during the first pollen season of year 1 commencing at approximately visit 6 did not induce relevant boosts of Bet v 1-specific IgG levels in the placebo group (Fig E2, B, and Fig E4). Bet v 1-specific IgG antibodies induced by vaccination with the fragments decreased after visit 7 and were low at visit 9 (week 48) when the fourth injection (ie, booster injection) was given (Fig E2, A, and Fig E4). For all actively treated patients with available serum samples after visit 9 (ie, A1, A2, A3, A5, A6), we found a strong and significant increase in Bet v 1- and fragment-specific IgG responses after the booster injection at visit 10 (week 60) (Fig E2, A, and Fig E4). No increases were found in the placebo-treated subjects at this time point. Only 1 placebo subject (ie, P8) showed a transient increase in Bet v 1-specific IgG at visit 11 after the birch pollen season (Fig E2, B), which may be explained by the fact that seasonal allergen exposure can boost allergen-specific IgG.⁷ Interestingly, Bet v 1-specific IgG levels, in particular IgG₄ levels, remained elevated in 3 (ie, A1, A2, and A3) of the 4 actively treated subjects with available serum samples even 48 weeks after the single booster injection at visit 13 (week 96) (Fig 1, A; Fig E2, A, and Fig E3). Competition ELISA experiments performed with sera from patients allergic to birch pollen (patients 1-11) (see this article's Methods section in the Online Repository at www.jacionline.org) showed that IgG antibodies induced by vaccination with rBet v 1 fragments in actively treated subjects, but not IgG antibodies from placebo-treated subjects, strongly inhibited IgE binding to Bet v 1 even at visit 13, suggesting that a sustained IgG antibody response blocking IgE binding to IgE epitopes of Bet v 1 was established (Fig 2). Bet v 1-specific IgG antibody responses in the actively treated subjects were accompanied by a Bet v 1-specific T-cell response and the induction of a mixed cytokine response consisting of T_H2 (eg, IL-4 and IL-5), tolerogenic (ie, IL-10), and T_H1 cytokines (IFN- γ) (see Fig E5, A and B, in this article's Online Repository at www.jacionline.org), which was not observed in the placebo group.

We also monitored the development of allergen-specific IgE antibodies as well as of skin sensitivity and compared it with allergen-specific IgG responses (Fig 1, B-F). After the first course of 3 vaccinations in year 1, 3 of the 6 actively treated subjects (ie, A1, A3, and A4) developed Bet v 1-specific IgE antibody levels slightly above the cutoff of the ImmunoCAP at visit 6 (week 12), which disappeared already at visit 7. After the booster injection given at visit 9, Bet v 1- and birch pollen-specific IgE levels

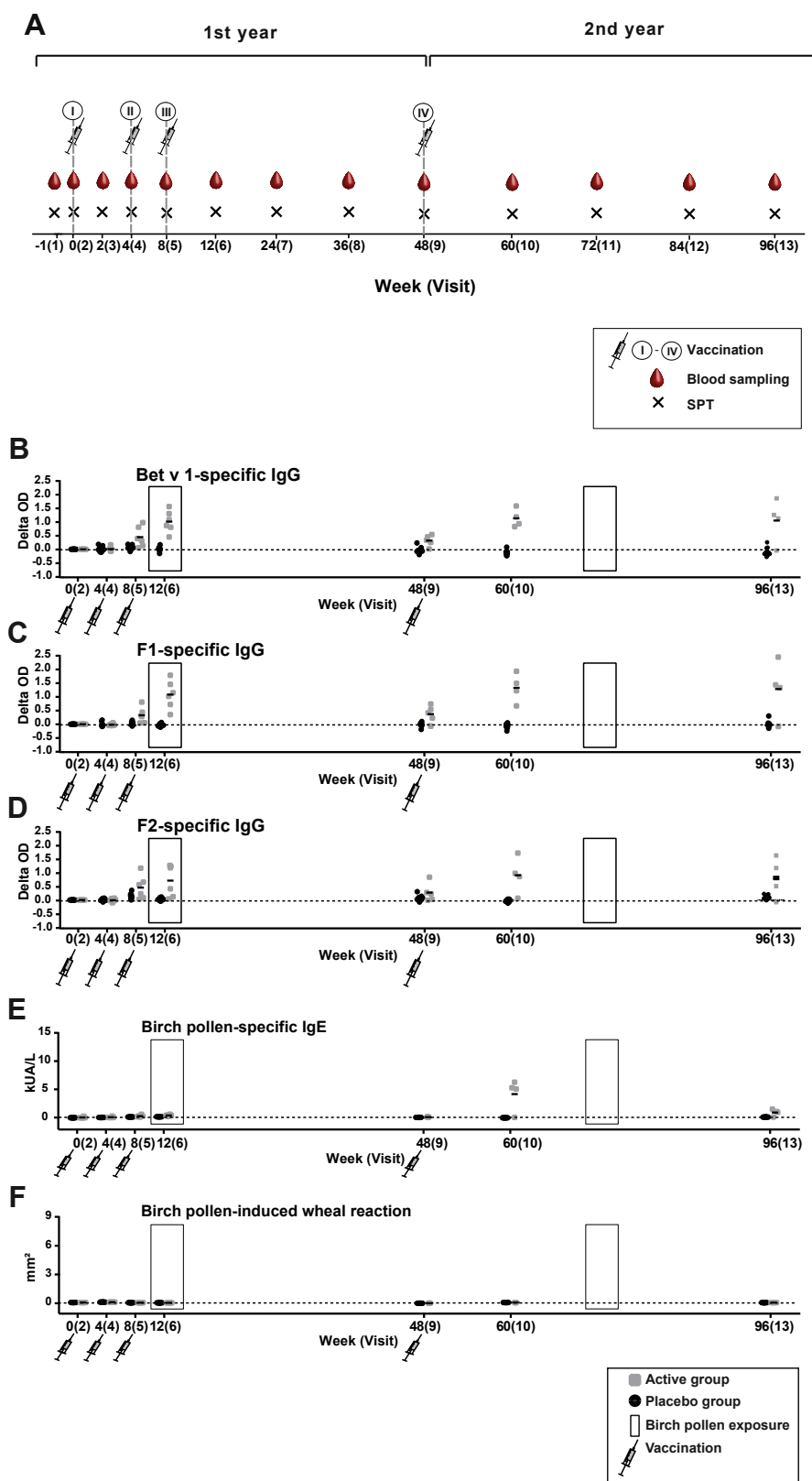


FIG 1. Scheme, time course of study, and development of specific IgG responses. **A**, The study was conducted over a period of 2 years (x-axis: weeks). The 13 study visits are indicated as well as the time points of vaccination (I-IV), blood sampling, and skin testing. Development of specific IgG responses (y-axis: OD increases compared with baseline) against, Bet v 1 (**B**), F1 (**C**), F2 (**D**) birch pollen-specific IgE (**E**) (y-axis: kU/L), and birch pollen-induced wheal reactions (**F**) (y-axis: mm²) during the 2 years of the study (x-axis: wk/visits) in the active group (grey) and the placebo group (black). Time points of vaccination and pollen seasons (boxed) are indicated. SPT, Skin prick test.

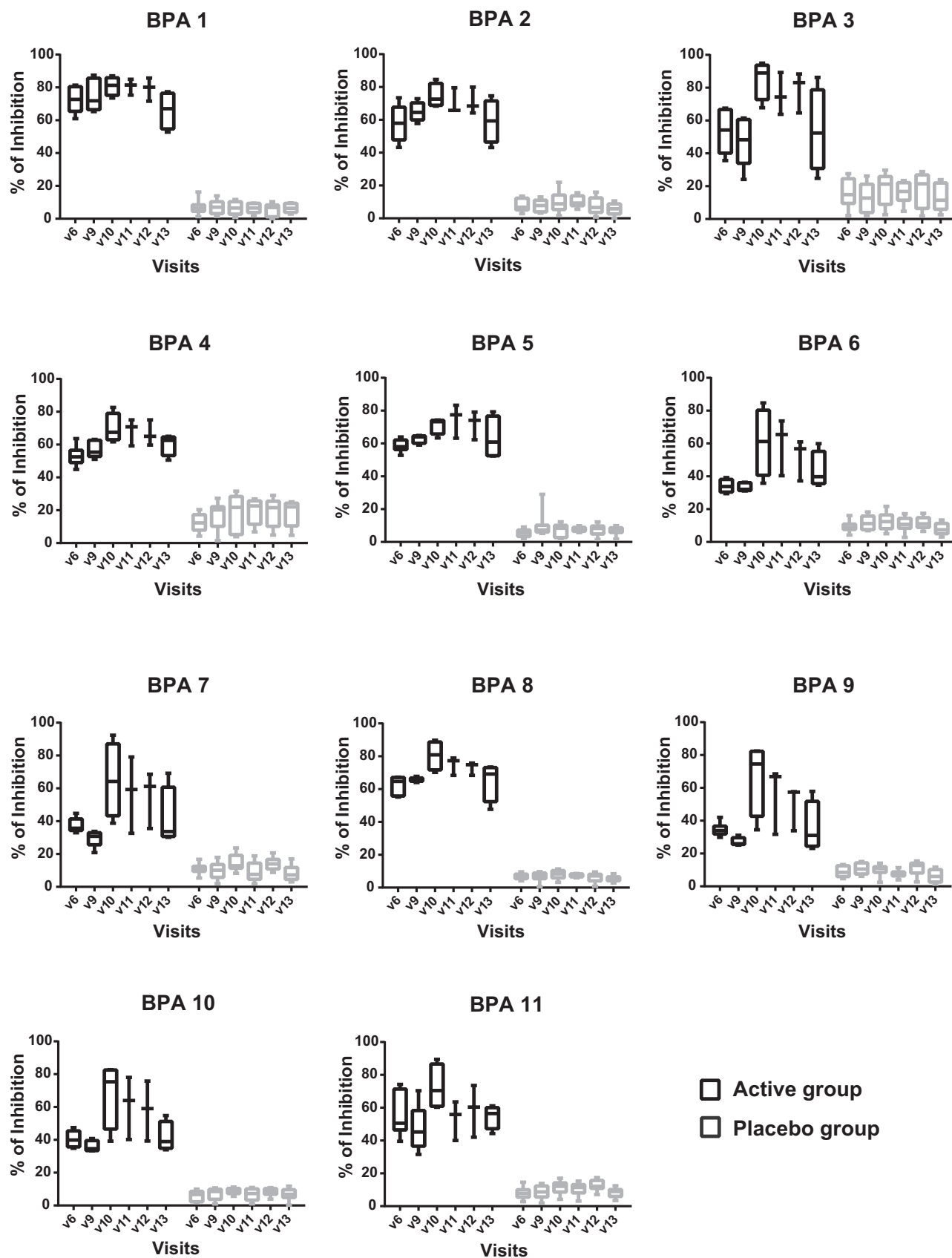


FIG 2. Inhibition of IgE binding to Bet v 1 by treatment-induced antibodies in patients with allergy. Shown are the percentages of inhibition as box plots with median and lower and upper quartiles of IgE binding of patients (BPA 1-11) obtained by preincubation of ELISA plate-bound Bet v 1 with sera from actively treated (black) or placebo-treated subjects (grey) taken at the different visits indicated (x-axes) (Table E5). BPA, Birch pollen allergic patient.

increased in 4 of the 5 actively treated subjects (ie, A1, A2, A3, and A5) for whom sera had been available. In contrast to Bet v 1-specific IgG levels, which remained high 48 weeks after the booster injection (ie, visit 13; week 96) (Fig 1, B, and Fig E2, A), birch pollen-specific IgE had strongly declined (Fig 1, E). Importantly, none of the actively treated subjects developed skin reactions to birch pollen at any time when tested by skin prick testing with birch pollen extract. The lack of birch pollen-specific skin reactions despite the presence of Bet v 1-specific IgE antibodies is most likely due to the protective effects of treatment-induced IgG antibodies, which were shown to also block IgE binding to Bet v 1 in patients with allergy (Fig 2). Thus, treatment of nonallergic subjects seems to have induced only clinically silent IgE sensitization. A summary of adverse events observed during the 2 years of the study is presented in Table E3 in this article's Online Repository at www.jacionline.org. These events were mild and classified as not related to the treatment. The only treatment-related side effects were mild (ie, local injection site reactions; n = 4), which occurred in 3 of the actively treated subjects (Table E3). Thus, vaccination of nonallergic subjects with recombinant hypoallergenic Bet v 1 derivatives was safe and well tolerated.

We are aware of only 1 other study in which a chemical conjugate consisting of a viral particle and a synthetic peptide derived from the major house dust mite allergen, Der p 1, was used to vaccinate nonallergic subjects.⁸ However, this study was conducted only over a period of 1 year and contained no placebo group, whereas our study reported that the synthetic vaccine induced allergen-specific IgG antibodies without inducing symptomatic allergic sensitization. One possible next first step could be to vaccinate children who have a clinically silent IgE sensitization and to assess whether this vaccination can prevent the development of allergic symptoms later in life. Our study is a small pilot study but may be considered as a first molecular approach toward preventive vaccination.

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REFERENCES

- Jacobsen L, Niggemann B, Dreborg S, Ferdousi HA, Halken S, Host A, et al. Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. *Allergy* 2007;62:943-8.
- Wickman M, Lupinek C, Andersson N, Belgrave D, Asarnoj A, Benet M, et al. Detection of IgE reactivity to a handful of allergen molecules in early childhood predicts respiratory allergy in adolescence. *EBioMedicine* 2017;26:91-9.
- Szpefalusi Z, Bannert C, Ronceray L, Mayer E, Hassler M, Wissmann E, et al. Preventive sublingual immunotherapy in preschool children: first evidence for safety and pro-tolerogenic effects. *Pediatr Allergy Immunol* 2014;25:788-95.
- Valenta R, Campana R, Marth K, van Hage M. Allergen-specific immunotherapy: from therapeutic vaccines to prophylactic approaches. *J Intern Med* 2012;272:144-57.
- Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest* 1997;99:1673-81.
- Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, et al. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci U S A* 2004;101:14677-82.
- Niederberger V, Ring J, Rakoski J, Jager S, Spitzauer S, Valent P, et al. Antigens drive memory IgE responses in human allergy via the nasal mucosa. *Int Arch Allergy Immunol* 2007;142:133-44.
- Kundig TM, Senti G, Schnetzler G, Wolf C, Prinz Vavricka BM, Fulurija A, et al. Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. *J Allergy Clin Immunol* 2006;117:1470-6.

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IgE-class-specific immunosuppression in offspring by administration of anti-IgE to pregnant mice



To the Editor:

The presence of atopic dermatitis is known to increase the risk of sensitization to food allergens. However, it is noteworthy that infants who present with atopic dermatitis before the age of 3 months have the highest risk of developing food allergies, and thereafter the risk decreases even if atopic dermatitis is present. Accumulating evidence also suggests that environmental events that occur during the first months of life may lead to "atopic march," that is, typical progression of various allergic diseases, through sensitization to aeroallergens as well as food allergens.¹ Reversal of specific IgE sensitization in adult patients does not seem realistic, due to development of memory immune cell types. Therefore, prevention of IgE sensitization in the first months of life can be thought to be the key to reducing the risk of future allergy development.

In the early 1980s, before the discovery of cytokines that control IgE production, IgE isotype-specific inhibition of antibody production was intensively investigated. According

Online Repository and supplemental information

Vaccination of non-allergic individuals with recombinant hypoallergenic fragments of birch pollen allergen Bet v 1: Safety, effects and mechanisms

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Supplemental Figures and Tables

FIG E1. Study population, enrolment and randomization. Discontinuations (numbers, reasons) are indicated.

Table E1. Demographic and clinical characterization of the study population.

FIG E2. Development of IgG responses against Bet v 1 and Bet v 1 fragments (F1, F2) (y-axes) during the study (x-axes: visits 1-13) in **A**, actively-treated (A1-A6) and **B**, placebo subjects (P1-P10).

FIG E3. Quantitative measurement of Bet v 1-specific IgG₁ and IgG₄ subclass levels. Shown are **A**, Bet v 1-specific IgG₁ and **B**, IgG₄ subclass levels in microgram per ml as box plots with median, lower and upper quartiles for the active and placebo group (y-axes) at different visits (x-axes). Significant differences are indicated (** p<0.01).

FIG E4. Bet v 1- and Bet v 1 fragment-specific IgG responses in actively and placebo-treated subjects at the different visits of the study. Shown are the geometric means of IgG levels as bars with SEM for available serum samples from the actively treated and placebo subjects for each visit. Significant differences are indicated (* p<0.05; ** p<0.01).

Table E2. Inhibition of allergic patients IgE binding to Bet v 1 by treatment-induced antibodies. Shown are percentages of inhibition of IgE binding of birch pollen allergic patients (BPA 1-11) obtained by pre-incubation of ELISA plate-bound Bet v 1 with sera from actively (A1-A6) or placebo-treated subjects (P1-P10) taken at the different visits indicated. Mean percentages $\pm$ SDs are shown for each visit for the actively and placebo-treated subjects.

FIG E5. Bet v 1-specific T cell and cytokine responses. Shown are **A**, Bet v 1-specific T cell responses (y-axes: stimulation indices, SIs) and in **B**, cytokine levels (IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-gamma, TNF-alpha, G-CSF, GM-CSF, MCP-1, MIP-1 β) (y-axes: pg/ml) as box plots with median, lower and upper quartiles for the active (black) and placebo group (grey) at different visits (x-axes). Significant differences are indicated (* $p < 0.05$; ** $p < 0.01$).

Table E3. Summary of adverse events, systemic and local reactions during the two years of the study.

MATERIALS AND METHODS

Study design

The clinical trial (ClinicalTrials.gov Identifier: NCT01353924) was conducted in the Vienna Challenge Chamber (VCC) from August 2012 until December 2014. The trial protocol and amendments were approved by the Ethics committee ("Ethikkommission der österreichischen Arbeitsgemeinschaft für klinische Pharmakologie und Therapie

und der privaten Krankenanstalt Institut für Hypertoniker, Vienna, Austria) and by the Austrian Health Agency (AGES, Vienna, Austria).

The trial was conducted as a single-center, double-blind, placebo-controlled trial in accordance with the principles of the Declaration of Helsinki (1964) (World Medical Association Declaration of Helsinki, 2012), including current revisions and in compliance with the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Uses (ICH's) Good Clinical Practice guidelines (ICH Guideline). Written informed consent was obtained from all participants.

Figure E1 provides a summary of screening, randomization and treatment of the subjects. In total 44 non-allergic male volunteers were screened of whom 17 individuals fulfilled all the criteria. The disposition of the study participants throughout the trial is summarized in Fig. 1. Inclusion and exclusion criteria were as follows:

Inclusion Criteria:

1. Age: 18 to 50 years
2. Male
3. No history of allergy
4. Negative skin prick tests for birch pollen and Bet v 1-fragments
5. Negative IgE for birch pollen and rBet v 1, mugwort pollen, house dust mite, cat, alder pollen, hazel pollen, timothy grass pollen
6. Healthy individuals according to history, physical examination and routine laboratory findings

7. Capable of giving informed consent, which includes compliance with the requirements and restrictions listed in the consent form

8. Available to complete the study

Exclusion criteria

1. History of drug or other allergy

2. Autoimmune disease, immune-defects including immuno-suppression, immune complex-induced immunopathies

3. Contra-indication for adrenaline

4. Long-term treatment with systemic corticosteroids, immunosuppressive drugs, tranquilizers or psychoactive drugs

5. Active tuberculosis

6. Multiple sclerosis

7. Severe psychological disorders

8. The subject has participated in a study involving an investigational drug within 90 days prior to visit 1

9. The subject is concurrently and within 6 months participating in another clinical study in which the subject is or will be exposed to an investigational or a non-investigational drug

10. The subject has donated a unit of blood (450ml) within the previous three months

11. Has a positive history for human immunodeficiency virus (HIV) antibodies or active hepatitis B or C

12. The subject is at risk of non-compliance with the study procedures/restriction

Vaccinations were performed three times in monthly intervals and one booster injection after one year. Monitoring of clinical and serological parameters was done for another year. Primary endpoint was the evolution of Bet v 1-, and Bet v 1-fragment-specific IgG antibody levels after vaccination with the Bet v 1 derivatives. A key secondary endpoint was to evaluate by skin prick testing if vaccination with hypoallergenic Bet v 1 derivatives induces allergic sensitization to natural, birch pollen-derived Bet v 1. Other secondary endpoints were the development of allergen-specific T cell and cytokine responses.

Investigational drug products

Bet v 1-derivatives (i.e., Bet v 1-fragments (fragment 1 representing amino acid 1-74 and fragment 2 representing amino acid 75- 160 of Bet v 1)) 14 produced in a standardized protocol under GMP conditions, were supplied by Biomay AG, Vienna, Austria. The vaccine was formulated in the hospital pharmacy of Salzburg by adsorption of the Bet v 1-derived fragments to sterile carbonate buffered Aluminium hydroxide (GE Healthcare, Little Chalfont, UK) following GMP guidelines. The active components of the vaccine were mixed in a 1:1 ratio. Carbonate-buffered Aluminium hydroxide alone was used as placebo. Vaccination was performed by subcutaneous injection of 200 µl of Bet v 1- fragment adsorbate containing 20 µg of each of each of

the two rBet v 1 fragments (i.e., F1, F2) or 200 µl placebo on the volunteer's lower third of the upper arm with a sterile syringe and needle.

For skin prick testing Bet v 1- fragments were bottled in equimolar amounts as stock solutions in 0.9% sodium chloride and diluted prior to use. Birch pollen extract registered for skin prick testing was purchased from Stallergenes, Antony, France. Glycerol-saline diluent as negative control solution and Histamine HCL 10 mg/ml as positive control solution were obtained from Stallergenes. Skin prick testing was performed as described in ^{E1}.

Determination of total and specific IgE antibodies

Total serum IgE levels and specific IgE levels to birch pollen extract and rBet v 1 were determined by ImmunoCAP measurements (Phadia, Uppsala, Sweden) as previously described ^{E2}.

Measurement of specific IgG levels

ELISA plates (Greiner, Kremsmünster, Austria) were coated (100µl/well) with rBet v 1 (2µg/ml), rBet v 1 F1 (1µg/ml), or rBet v 1 F2 (1µg/ml) diluted in carbonate buffer, pH 9.6 for 5 hours at room temperature. The detection of specific IgG antibodies was performed as described ^{E2} using a plate-to-plate normalization ^{E3}.

Quantification of allergen-specific IgG₁ and IgG₄ levels

The quantification of allergen-specific IgG₁ and IgG₄ subclass levels was performed by ELISA as described ^{E3}. Quantification of allergen-specific IgG₁ and IgG₄ was achieved by using a purified human monoclonal Phl p 2-specific IgG₁ antibody ^{E4} and the corresponding recombinant Phl p 2 allergen, as well as a purified human monoclonal Phl p 5-specific IgG₄ antibody ^{E5} and the corresponding recombinant Phl p 5 allergen for establishing standardization curves which allowed to calculate the levels of specific IgG₁ and IgG₄ in microgram/ml (Weber M., & Valenta R, unpublished).

Measurement of antibodies capable of blocking allergic patients allergen-specific IgE binding

ELISA plates (Greiner, Kremsmünster, Austria) were coated with 100µl of rBet v 1 (1µg/ml in carbonate buffer, pH 9.6) for 5 hours at room temperature. Plates were blocked with 2% bovine serum albumin (BSA) (Roth, Karlsruhe, Germany) in PBS-T (PBS 0.05% Tween 20) overnight at 4°C. ELISA plate-bound rBet v 1 was pre-incubated with heat-treated sera (1.5 hours, 56°C) (1:1) from the vaccinated subjects overnight at 4°C. Plates were washed 3 times with PBS-T and then exposed to sera from eleven different birch pollen allergic patients (1:5). After overnight incubation at 4°C, plates were washed 5 times with PBS-T and bound human IgE antibodies were detected using a 1:2500 diluted Goat anti-human IgE-HRP antibody (KPL, Milford, MA) for 1 hour at 37°C and 4°C. Plates were again washed 5 times with PBS-T and colour reaction was determined as described ^{E6}. The percentage of inhibition of IgE-binding was calculated using the OD values obtained, as follows: percent inhibition of IgE binding = 100 – (OD different visits/OD baseline visit 2) x 100.

186

187 Lymphocyte proliferation assays and detection of secreted cytokines

188 Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood
189 samples by Ficoll density-gradient separation (Amersham, GE Healthcare,
190 Buckinghamshire, UK). PBMC were then stimulated with 1 µg/well of rBet v 1 or with
191 an equimolar mix of hypoallergenic rBet v 1 fragments (F1+F2) in triplicates at a
192 density of 2×10^6 cells/well in 96-well round-bottom plates (Thermo Fischer Scientific,
193 Roskilde, Denmark). Cells were cultured in 200 µL supplemented Ultra Culture
194 Medium (Lonza, Verviers, Belgium) as described ^{E6}. Supernatants from equally
195 prepared PBMCs cultures were harvested at day 6 and levels of IL-1, IL-2, IL-4, IL-5,
196 IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, INF-γ, TNF-α, C-GSF, GM-CSF, MCP-1,
197 and MIP-1b were measured using Bio-Plex Pro TM human cytokine 17-plex
198 immunoassay (Bio-Rad Inc., Hercules, CA) as described ^{E6}.

199

200

201 Statistical analysis of data

202 The differences between the randomized groups were assessed by T-test (i.e., age,
203 height, weight) and Mann-Whitney test (i.e., Total IgE). In addition, geometric means
204 of IgG antibody levels were computed for each treatment group and time point
205 together with 95% confidence intervals. A *P* value < 0.05 was considered as
206 statistically significant.

207

208

References

- E1. Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, et al. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci U S A* 2004; 101 Suppl 2:14677-82.
- E2. Ziegelmayer P, Focke-Tejkl M, Schmutz R, Lemell P, Ziegelmayer R, Weber M, et al. Mechanisms, safety and efficacy of a B cell epitope-based vaccine for immunotherapy of grass pollen allergy. *EBioMedicine* 2016; 11:43-57.
- E3. Stern DA, Riedler J, Nowak D, Braun-Fahrlander C, Swoboda I, Balic N, et al. Exposure to a farming environment has allergen-specific protective effects on TH2-dependent isotype switching in response to common inhalants. *J Allergy Clin Immunol.* 2007; 119:351-8.
- E4. Flicker S, Steinberger P, Norderhaug L, Sperr WR, Majlesi Y, Valent P, Kraft D, Valenta R. Conversion of grass pollen allergen-specific human IgE into a protective IgG(1) antibody. *Eur J Immunol.* 2002;32:2156-62.
- E5. Madritsch C, Flicker S, Scheiblhofer S, Zafred D, Pavkov-Keller T, Thalhamer J, et al. Recombinant monoclonal human immunoglobulin E to investigate the allergenic activity of major grass pollen allergen Phl p 5. *Clin Exp Allergy.* 2011;41:270-80.
- E6. Campana R, Moritz K, Neubauer A, Huber H, Henning R, Brodie TM, et al. Epicutaneous allergen application preferentially boosts specific T cell responses in sensitized patients. *Sci Rep.* 2017;7:11657. doi: 10.1038/s41598-017-10278-1.

Table E1

	Bet v 1a F1/F2	Placebo	P value
Number of Subjects	6	10	
Age (years)			
Mean	30.3	27.7	0.589
SD	9.7	9.0	
Height (cm)			
Mean	177.0	178.6	0.533
SD	4.4	5.1	
Weight (Kg)			
Mean	71.0	81.9	0.072
SD	7.4	12.3	
Total IgE (kUA/L)			
Mean	43.4	42.5	0.278
SD	32.0	56.3	
Gender			
Male (%)	6 (100)	10 (100)	
Ethnic group (%)			
Caucasian	5 (83.3)	10 (100)	
Asian	0	0	
African	0	0	
Other	1 (16.7)	0	
Family history of allergy			
Parents	0	0	
Siblings	1	1	
Birch (kUA/L)			
Mean	<0.35	<0.35	
House dust mite (kUA/L)			
Mean	<0.35	<0.35	
Mugwort (kUA/L)			
Mean	<0.35	<0.35	
Cat (kUA/L)			
Mean	<0.35	<0.35	
Grass pollen (kUA/L)			
Mean	<0.35	<0.35	

Table E2

Visits	BPA 1																			
	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	60.98	72.40	73.00	66.98	81.40	80.16	72.49	7.76	16.28	3.27	1.30	5.00	6.92	7.58	6.07	10.99	6.83	5.13	6.94	4.18
v9	71.89	65.31	67.64	n.d.	83.74	87.46	75.21	9.86	1.44	1.67	3.65	5.29	10.63	8.47	5.64	10.30	9.06	13.92	7.01	4.13
v10	n.d.	73.53	82.25	n.d.	80.39	87.06	80.80	5.61	5.35	1.88	0.76	7.79	10.13	10.98	4.26	11.73	10.13	3.06	6.61	4.05
v11	n.d.	75.31	81.43	n.d.	n.d.	84.94	80.56	4.87	3.58	4.30	1.71	7.03	6.06	9.49	n.d.	9.41	9.29	n.d.	6.36	2.97
v12	n.d.	71.59	80.11	n.d.	n.d.	85.73	79.14	7.12	0.25	3.08	1.51	7.57	8.96	7.52	0.02	8.14	10.38	6.30	5.37	3.81
v13	60.39	73.67	77.36	n.d.	n.d.	52.77	66.05	11.47	4.14	2.84	3.01	4.56	6.55	6.36	6.33	9.32	9.62	9.43	6.22	2.59
Visits	BPA 2																			
	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	54.42	73.48	49.34	43.22	65.80	61.57	57.97	11.13	6.43	12.21	13.50	4.39	6.88	7.37	2.90	12.47	6.63	5.32	7.81	3.65
v9	62.08	72.88	57.82	n.d.	64.54	67.94	65.05	5.72	5.18	13.06	12.75	3.20	8.59	6.96	3.42	4.13	9.37	10.46	7.71	3.71
v10	n.d.	84.65	68.40	n.d.	74.96	70.21	74.55	7.28	7.19	14.86	14.04	5.72	10.14	21.94	4.28	7.54	12.21	1.61	9.95	5.99
v11	n.d.	79.56	65.82	n.d.	n.d.	65.76	70.38	7.95	7.57	11.76	14.39	5.50	9.24	15.62	n.d.	9.32	8.15	n.d.	10.19	3.47
v12	n.d.	79.98	64.24	n.d.	n.d.	68.49	70.91	8.14	6.46	12.19	13.33	4.44	6.58	15.93	1.32	0.75	10.19	6.67	7.79	5.05
v13	43.16	74.66	62.24	n.d.	n.d.	56.46	59.13	13.08	4.97	2.80	10.61	2.44	8.63	9.42	0.07	4.86	6.54	7.61	5.79	3.38
Visits	BPA 3																			
	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	43.70	35.65	67.50	41.59	64.78	66.41	53.27	14.46	14.54	2.16	13.34	11.70	23.77	18.60	2.90	27.63	26.81	15.19	15.66	8.88
v9	43.75	24.15	48.28	n.d.	59.62	61.56	47.47	15.04	12.44	2.18	20.73	10.10	26.19	13.17	2.22	22.70	17.06	4.59	13.14	8.54
v10	n.d.	89.91	94.95	n.d.	87.99	67.77	85.15	11.96	18.66	6.54	20.33	9.74	25.89	25.96	2.64	29.75	22.19	25.58	18.73	9.28
v11	n.d.	74.41	89.32	n.d.	n.d.	63.82	75.85	12.81	20.14	4.67	16.26	11.62	23.42	17.25	n.d.	22.80	11.38	n.d.	15.94	6.43
v12	n.d.	83.10	88.40	n.d.	n.d.	64.64	78.71	12.48	21.24	7.60	22.81	11.36	26.54	28.87	3.12	27.78	21.72	1.91	17.29	10.34
v13	48.67	24.79	86.31	n.d.	n.d.	56.00	53.94	25.36	21.67	3.90	12.76	8.49	23.95	22.79	2.56	15.34	10.19	6.62	12.83	7.86

BPA 4																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	54.18	52.40	63.60	44.90	52.64	50.38	53.02	6.11	8.28	12.55	4.18	19.05	20.41	14.53	12.02	16.70	11.70	6.82	12.62	5.24
v9	63.10	51.07	62.09	n.d.	54.50	55.35	57.22	5.17	6.69	11.36	1.42	21.33	27.31	21.94	19.97	22.81	20.38	16.11	16.93	8.06
v10	n.d.	82.63	68.36	n.d.	66.54	61.72	69.81	8.99	8.98	3.86	4.39	20.20	31.66	24.47	27.89	29.95	23.17	5.43	18.00	11.18
v11	n.d.	75.00	70.83	n.d.	n.d.	59.17	68.33	8.21	10.75	13.52	6.77	25.19	25.75	26.91	n.d.	25.66	20.04	n.d.	19.32	7.92
v12	n.d.	75.04	65.00	n.d.	n.d.	59.70	66.58	7.79	11.30	7.01	4.87	18.24	29.02	24.92	26.09	24.78	25.21	14.49	18.59	8.69
v13	63.52	61.60	65.01	n.d.	n.d.	50.53	60.17	6.57	10.86	4.69	9.31	23.41	21.58	22.58	24.97	25.09	24.08	15.88	18.24	7.50
BPA 5																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	57.45	63.97	61.51	52.78	58.48	57.65	58.64	3.83	6.45	9.11	8.77	6.47	6.81	4.30	2.01	1.83	3.88	3.82	5.34	2.57
v9	64.84	64.39	64.02	n.d.	59.06	61.19	62.70	2.48	7.52	11.20	7.72	6.31	9.83	6.53	29.05	5.20	7.00	5.34	9.57	7.10
v10	n.d.	73.17	73.44	n.d.	74.30	63.36	71.07	5.16	9.73	12.28	9.03	7.75	11.22	7.19	1.61	2.33	9.24	2.82	7.32	3.80
v11	n.d.	77.45	83.33	n.d.	n.d.	63.17	74.65	10.37	8.83	9.86	8.92	7.31	8.15	6.32	n.d.	5.71	8.41	n.d.	7.94	1.40
v12	n.d.	74.11	79.12	n.d.	n.d.	62.32	71.85	8.63	8.02	12.17	10.38	7.98	8.68	8.63	1.50	4.73	9.52	4.94	7.65	3.12
v13	53.02	68.81	79.33	n.d.	n.d.	52.38	63.38	13.07	5.86	9.97	7.60	6.31	7.56	7.31	1.83	5.53	8.71	8.65	6.93	2.26
BPA 6																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	37.82	39.20	32.40	35.26	30.69	29.44	34.13	3.94	8.04	4.21	9.60	8.40	16.14	7.81	7.23	9.27	14.76	9.31	9.48	3.51
v9	32.06	35.88	36.39	n.d.	31.70	31.11	33.43	2.50	7.12	8.29	9.72	7.63	18.25	12.64	6.89	13.02	15.71	16.13	11.54	4.18
v10	n.d.	66.99	84.69	n.d.	55.35	35.75	60.70	20.54	9.91	11.03	17.22	8.87	21.69	13.71	5.73	14.50	16.16	5.04	12.39	5.26
v11	n.d.	65.49	73.67	n.d.	n.d.	40.41	59.86	17.33	8.03	2.76	14.45	9.25	17.21	10.23	n.d.	11.23	14.14	n.d.	10.91	4.48
v12	n.d.	56.75	60.94	n.d.	n.d.	37.22	51.64	12.66	8.98	9.21	15.02	10.29	17.48	11.74	6.44	8.63	14.77	12.63	11.52	3.45
v13	34.63	41.18	59.84	n.d.	n.d.	38.33	43.50	11.22	5.08	3.04	10.84	8.14	13.35	6.73	4.91	6.55	12.60	8.77	8.00	3.41

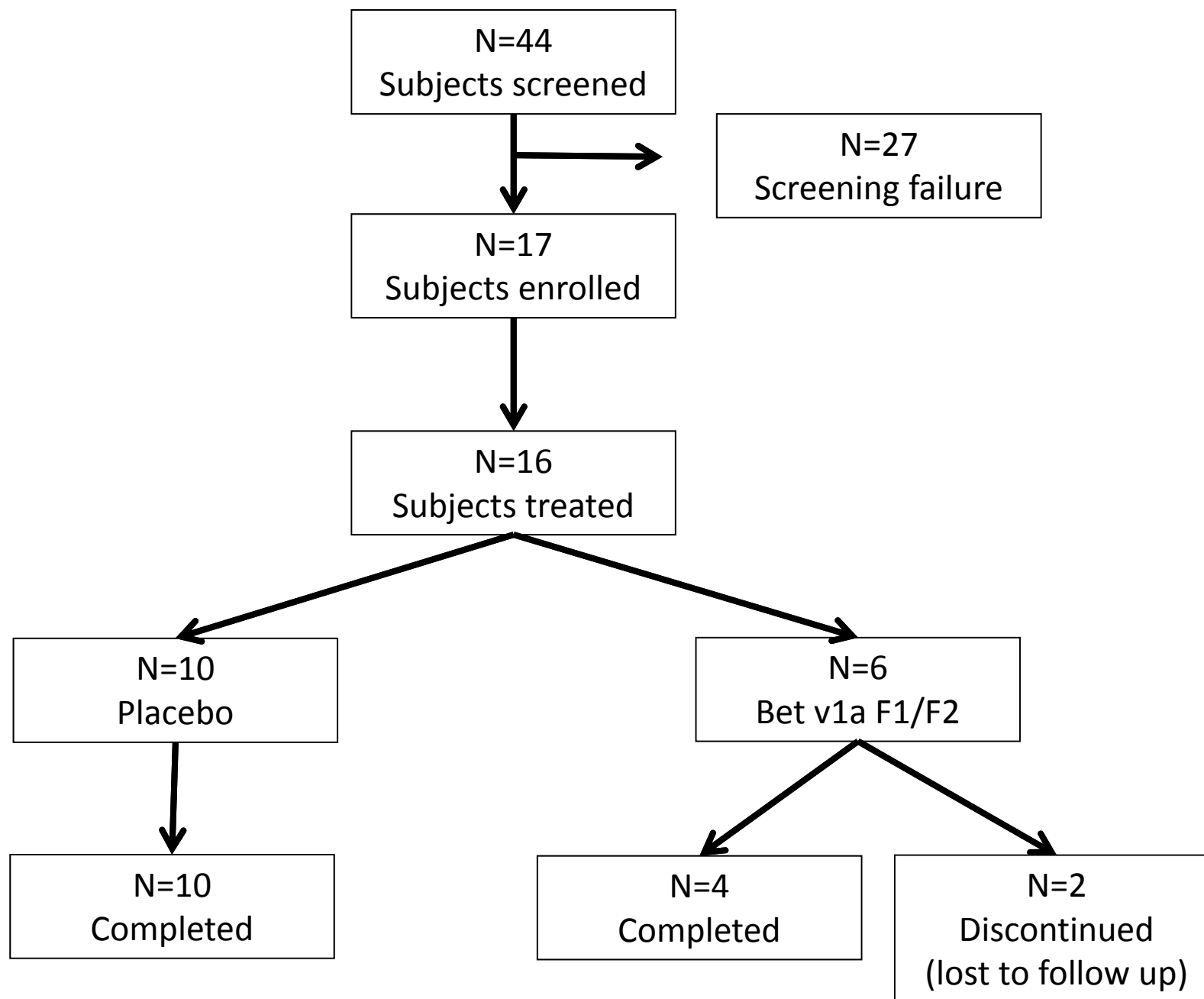
BPA 7																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	40.21	35.55	44.77	35.94	34.81	32.92	37.37	4.35	11.63	10.64	11.80	10.68	16.75	8.17	9.85	14.86	5.34	11.35	11.11	3.17
v9	33.77	20.88	30.81	n.d.	32.10	30.34	29.58	5.04	4.83	8.60	13.24	9.99	17.92	10.32	6.06	14.68	1.97	11.56	9.92	4.79
v10	n.d.	56.83	92.34	n.d.	71.52	38.82	64.88	22.67	9.56	17.46	17.79	11.95	23.42	11.98	13.38	23.73	12.30	8.33	14.99	5.41
v11	n.d.	59.28	79.16	n.d.	n.d.	32.62	57.02	23.35	5.97	4.44	5.11	9.32	18.84	10.18	n.d.	15.88	1.91	n.d.	8.96	5.87
v12	n.d.	61.21	68.61	n.d.	n.d.	35.54	55.12	17.36	8.76	15.65	15.83	11.25	19.18	14.00	9.72	20.72	13.93	12.05	14.11	3.87
v13	32.19	35.26	69.22	n.d.	n.d.	30.19	41.71	18.46	3.07	7.55	8.63	3.14	17.01	10.93	4.99	14.50	5.14	7.01	8.20	4.69
BPA 8																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	64.02	65.63	65.49	n.d.	65.80	67.82	65.75	1.36	4.66	8.90	7.23	9.60	5.95	7.56	5.74	7.98	6.56	4.13	6.83	1.77
v9	n.d.	85.37	89.80	n.d.	76.30	70.22	80.42	8.82	3.76	9.40	7.56	6.76	9.73	8.60	6.06	8.72	6.96	0.54	6.81	2.83
v10	n.d.	77.25	78.95	n.d.	n.d.	68.34	74.85	5.70	5.14	10.56	9.01	6.88	11.34	10.29	6.26	10.07	7.67	3.17	8.04	2.67
v11	n.d.	75.90	74.86	n.d.	n.d.	68.36	73.04	4.08	5.93	7.03	7.00	7.95	8.66	8.44	n.d.	8.21	6.43	n.d.	7.46	1.00
v12	72.22	66.19	73.46	n.d.	n.d.	47.73	64.90	11.88	3.85	5.04	8.09	6.45	8.15	9.57	4.27	7.84	8.10	0.82	6.22	2.68
v13	43.16	74.66	62.24	n.d.	n.d.	56.46	59.13	13.08	2.79	8.49	4.47	5.13	6.18	6.83	4.98	6.44	5.97	3.37	5.47	1.69
BPA 9																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	33.29	29.89	34.68	42.05	32.57	34.93	34.57	4.09	9.04	12.48	12.62	7.96	4.71	6.38	4.80	7.93	9.39	13.28	8.86	3.14
v9	31.12	25.93	25.99	n.d.	25.09	28.14	27.25	2.44	10.16	7.22	15.13	6.15	7.66	11.26	8.69	13.98	10.86	15.10	10.62	3.27
v10	n.d.	81.74	82.48	n.d.	67.34	34.45	66.50	22.47	9.02	10.34	12.34	10.03	11.83	11.58	2.45	14.15	11.70	7.08	10.05	3.30
v11	n.d.	68.53	66.89	n.d.	n.d.	31.74	55.72	20.78	11.38	8.91	3.94	7.42	5.99	8.42	n.d.	7.68	7.82	n.d.	7.69	2.16
v12	n.d.	57.72	57.41	n.d.	n.d.	33.88	49.67	13.68	12.42	14.11	14.10	7.83	2.69	13.59	8.22	13.10	10.94	15.43	11.24	3.92
v13	23.11	28.63	57.88	n.d.	n.d.	33.44	35.77	15.34	2.80	4.89	2.10	1.41	5.51	10.09	6.59	10.40	7.25	11.73	6.28	3.62

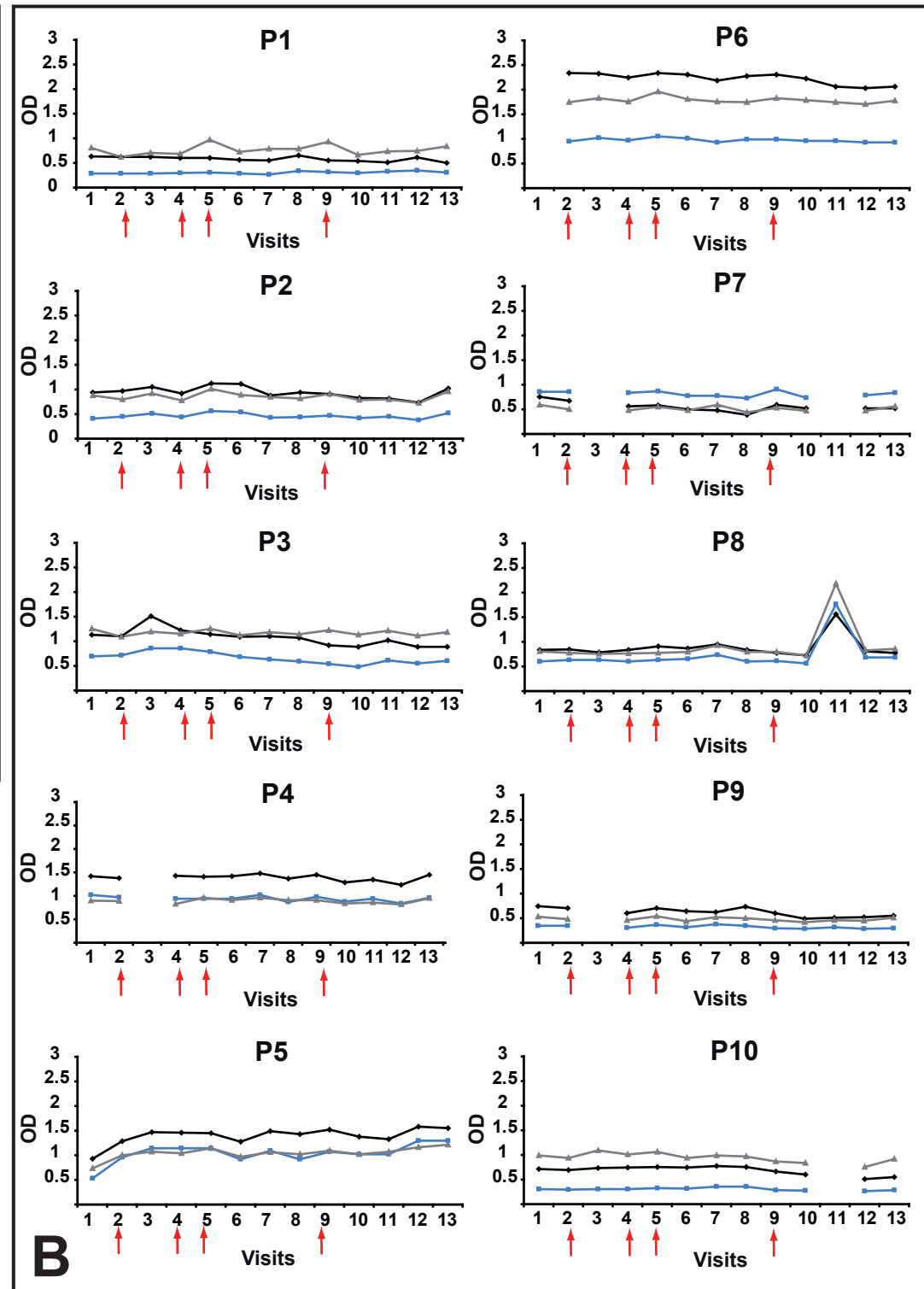
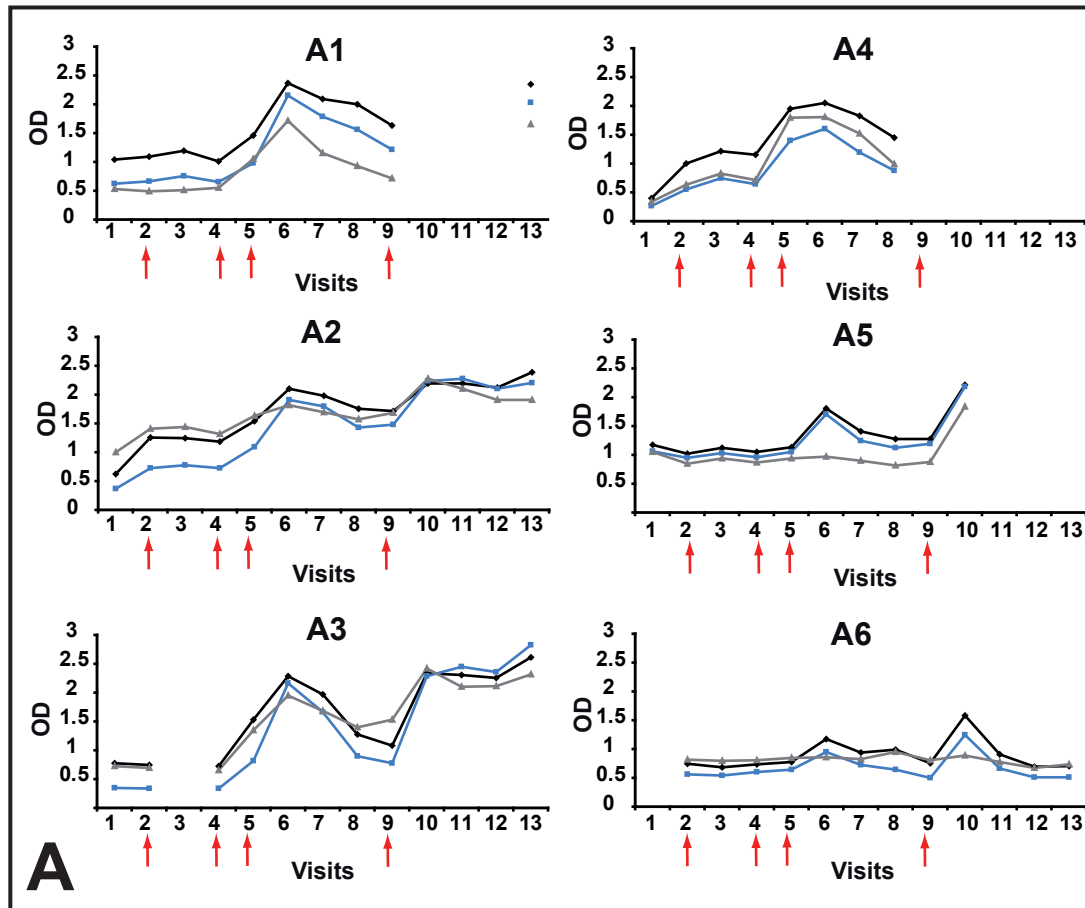
BPA 10																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	47.44	34.88	35.94	38.02	44.82	41.65	40.46	5.03	6.73	9.88	1.70	6.30	2.32	2.31	7.19	8.19	6.76	9.96	6.13	3.05
v9	40.82	33.54	33.33	n.d.	38.31	34.60	36.12	3.30	9.09	9.51	2.20	4.28	0.97	5.87	8.01	10.19	10.74	9.75	7.06	3.52
v10	n.d.	82.20	82.67	n.d.	68.58	39.19	68.16	20.39	10.87	7.50	11.20	6.64	5.62	9.08	8.74	10.02	10.21	8.53	8.84	1.83
v11	n.d.	78.00	63.99	n.d.	n.d.	40.24	60.74	19.09	9.54	10.93	1.03	5.97	2.71	5.19	n.d.	8.60	10.28	n.d.	6.78	3.65
v12	n.d.	75.80	59.05	n.d.	n.d.	39.29	58.05	18.27	5.22	8.59	8.49	7.47	8.21	9.43	10.88	10.81	10.09	3.87	8.30	2.29
v13	40.43	33.95	54.81	n.d.	n.d.	37.55	41.69	9.14	4.91	9.60	4.90	5.22	0.22	9.66	8.41	8.66	11.82	4.82	6.82	3.40
BPA 11																				
Visits	A1	A2	A3	A4	A5	A6	Mean %	SD	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean %	SD
v6	70.41	51.81	74.21	48.73	49.26	39.54	55.66	13.60	9.72	6.75	3.59	10.84	2.82	6.21	8.45	8.73	7.06	14.62	7.88	3.45
v9	46.24	45.17	70.38	n.d.	41.53	31.50	46.96	14.32	12.33	6.65	1.92	13.86	7.42	8.66	11.46	8.69	2.40	12.29	8.57	4.11
v10	n.d.	78.18	89.49	n.d.	60.29	62.77	72.68	13.72	17.03	13.17	13.67	11.72	12.50	10.01	9.44	15.95	4.17	6.29	11.39	4.03
v11	n.d.	63.51	55.88	n.d.	n.d.	40.06	53.15	11.96	15.30	11.96	9.48	13.17	10.97	7.71	n.d.	12.96	3.09	n.d.	10.58	3.82
v12	n.d.	73.58	60.37	n.d.	n.d.	42.06	58.67	15.83	16.65	17.45	11.70	13.39	12.55	11.67	11.56	15.34	7.14	8.85	12.63	3.24
v13	56.05	56.85	61.10	n.d.	n.d.	44.28	54.57	7.21	12.49	10.18	5.64	9.25	6.09	9.29	8.60	10.07	3.43	6.68	8.17	2.67

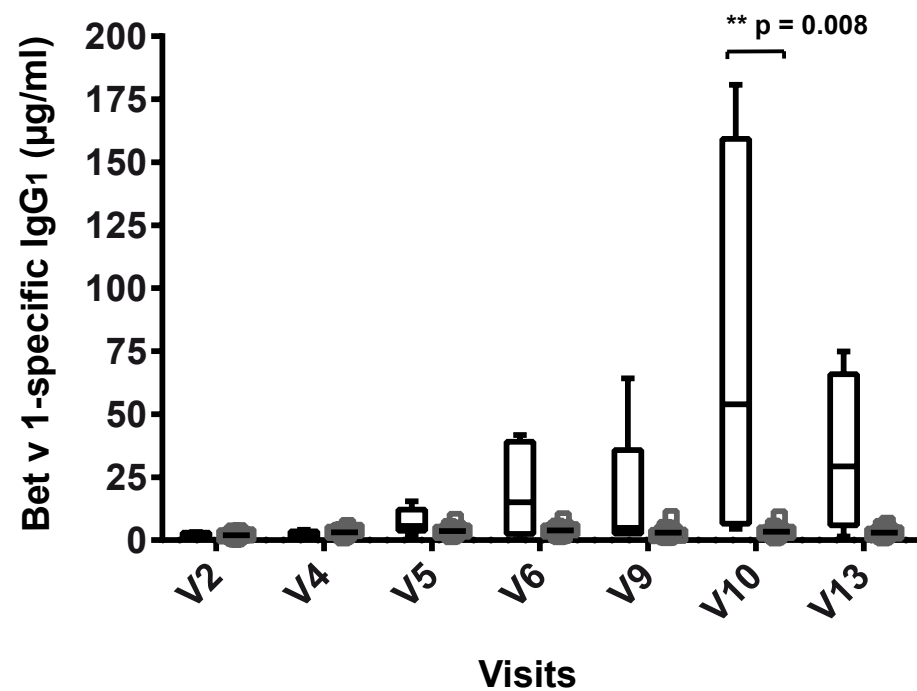
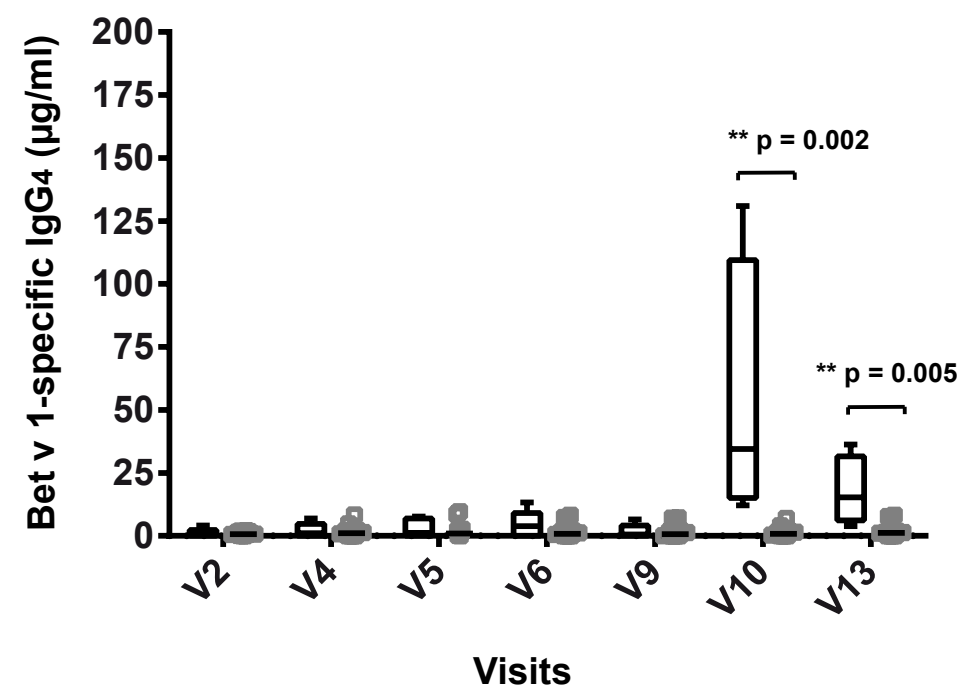
Table E3

Event	Placebo	Bet v 1 F1/F2
(No of events / No of patients)		
Adverse event		
Any	(36 / 10)	(16 / 5)
Related	0	0
SAE	0	0
Local drug reaction	0	(4 / 3)

Figure E1

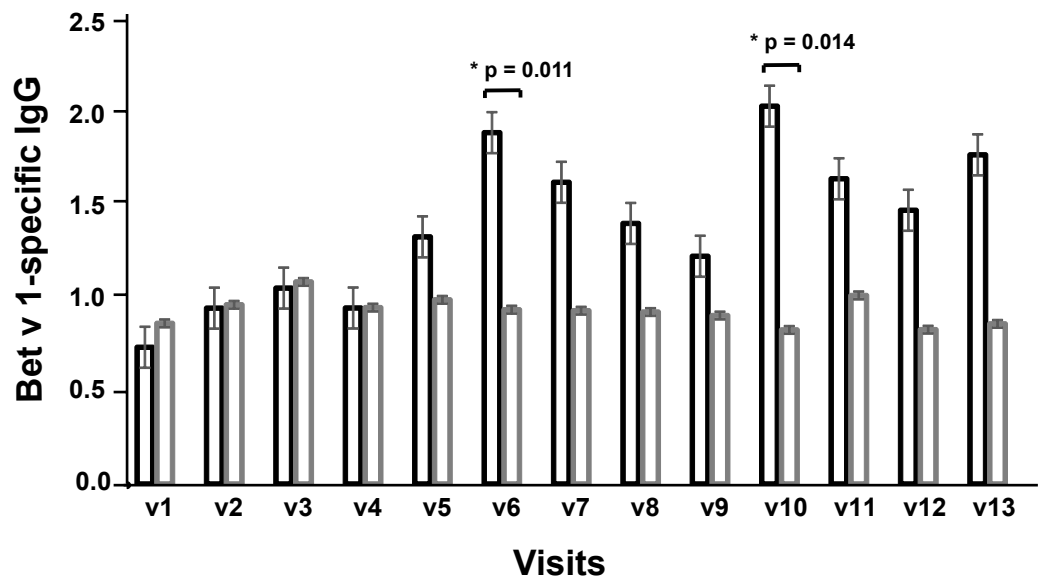




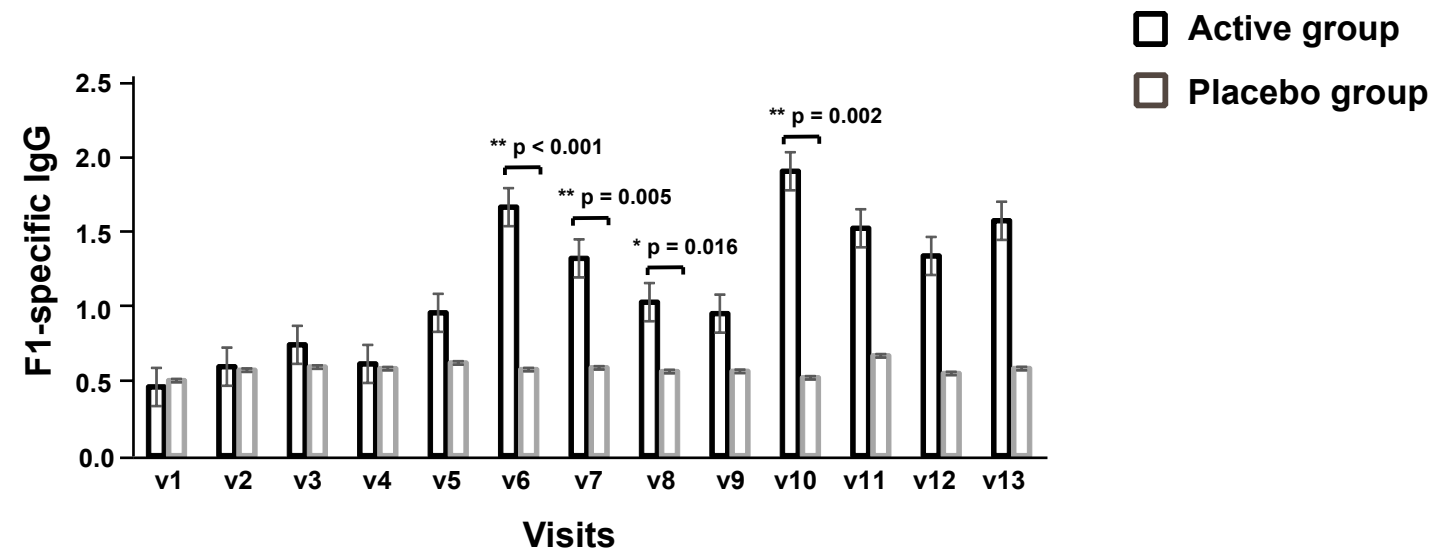
A**B**

□ Active group
■ Placebo group

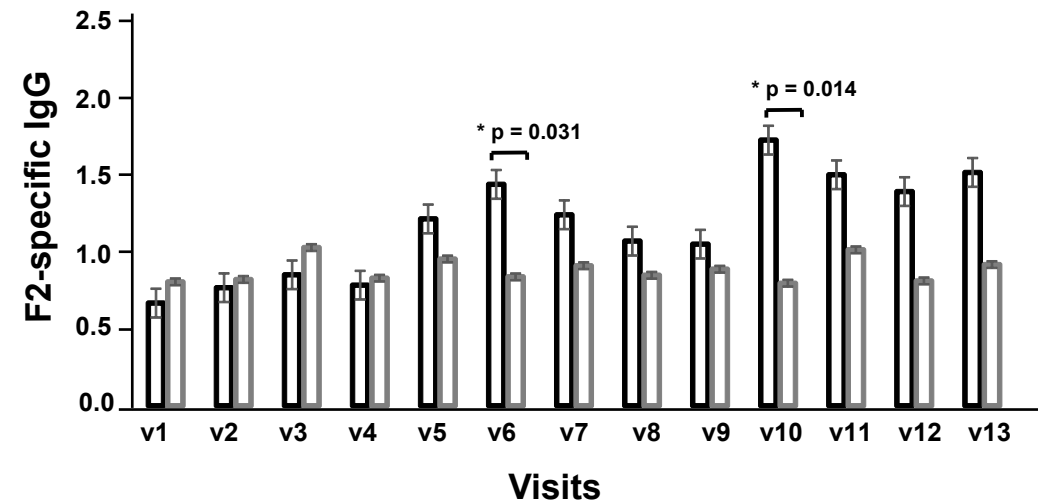
A

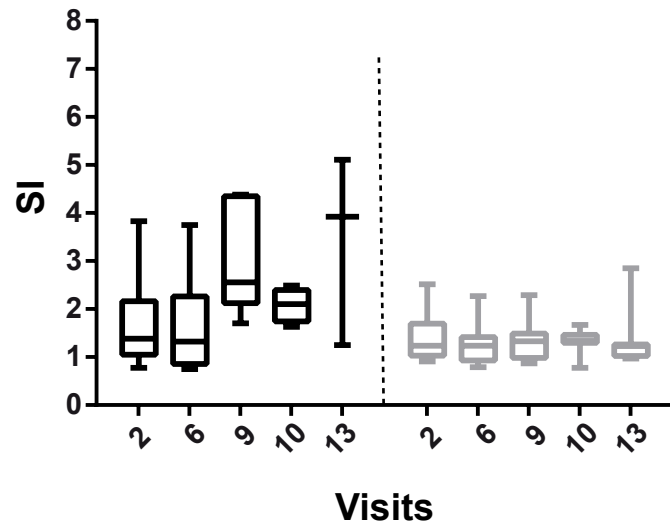
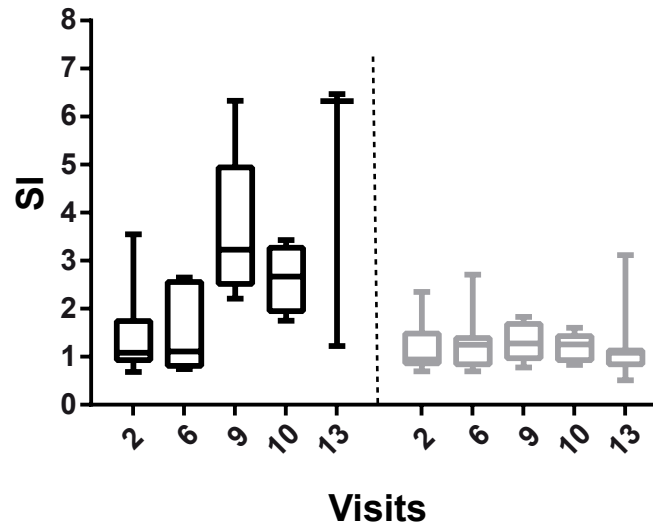
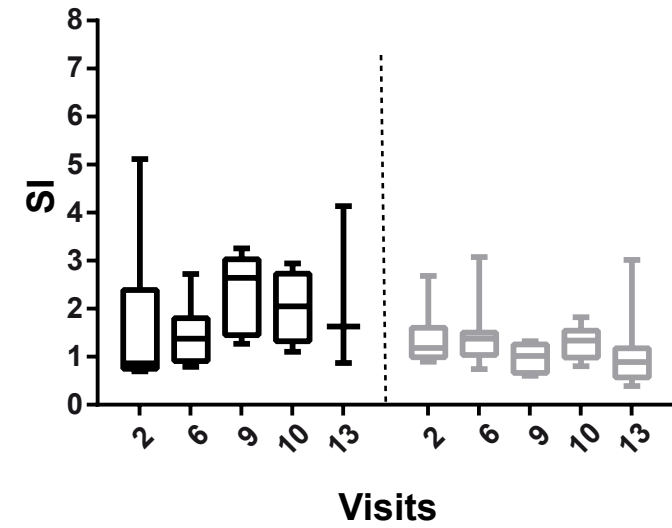


B



C



rBet v 1**F1****F2**

□ Active group
□ Placebo group

