

Identification of Seasonal Variations of Antibodies against PR-10-Specific Epitopes Can Be Improved Using Peptide-Phage Display

Lisbeth Ramírez Caballero^a Christoph Kny^a Regina Treudler^b Jan C. Simon^b
Karolin Kern^a Uta Jappe^{c,d} Michael Szardenings^a

^aLigand Development Unit, Fraunhofer Institute of Cell Therapy and Immunology, Leipzig, Germany; ^bLeipzig Comprehensive Allergy Center LICA-CAC, Department of Dermatology, Venereology and Allergology, University of Leipzig, Leipzig, Germany; ^cDivision of Clinical and Molecular Allergology, Research Center Borstel, Airway Research Center North (ARCN), German Center for Lung Research, Borstel, Germany; ^dInterdisciplinary Outpatient Clinic, Department of Pneumology, University of Lübeck, Lübeck, Germany

Keywords

Allergen exposure · Birch pollen allergy · Epitope · Epitope mapping · Hazelnut · Phage display · Pollen seasons · Pollinosis

Abstract

Background: In pollinosis patients, allergen-specific antibody titers show seasonal variations. Little is known about these variations at the epitope level. **Objectives:** We aimed at investigating seasonal variations on the level of allergen epitope recognition in patients with Bet v 1-related food allergy using a peptide phage display approach. **Methods:** Serum samples collected over 1 year from 4 patients of the placebo arm of the birch-associated soya allergy immunotherapy trial were included. To identify epitopes from Bet v 1-related food allergens, patient sera were used in peptide phage display experiments. In silico analysis of enriched allergen-related motifs was performed. **Results:** We identified epitope motifs related to Bet v 1 and its homologs in soya and hazelnut (Gly m 4 and Cor a 1, respectively) that were enriched in accordance with birch and hazel pollen exposure. Within several weeks after the birch pollen season peak, the pattern of identified epitope motifs differed considerably among patients. Data for amino acid preferences in homologous Bet v 1 and

Cor a 1 epitope motifs identified for one of the investigated patients suggest changes in concentration or specificity of serum antibodies for the Cor a 1 epitope motif. **Conclusions:** Peptide phage display data suggest an impact of birch and hazel pollen exposure on the recognition pattern of Bet v 1-like allergen epitopes. Epitope-oriented analyses could provide deeper, personalized details regarding the allergen epitope recognition influenced by pollen exposure beyond the capability of current methods.

© 2020 S. Karger AG, Basel

Introduction

Seasonal variations of birch pollen exposure have an impact on allergen-specific antibody levels of affected patients [1–3]. This was also observed within the study on birch-associated soya allergy immunotherapy (BASALIT), which investigated the clinical benefits of allergen-specific immunotherapy (AIT) using a recombinant folding variant of the major birch pollen allergen Bet v 1 in birch-related soya allergy [4]. Measurements of allergen-specific antibody concentrations are useful during allergy diag-

Edited by: H.-U. Simon, Bern.

Table 1. Overview of serum collection dates from investigated BASALIT placebo patients

Patient ID	Gender (M/F); age, years	Serum collection date (DD/MM/YY)				
		t_1 , autumn	t_2 , late autumn	t_3 , winter/spring	t_4 , spring/summer	t_5 , autumn
P1	M; 27	01/10/12	28/11/12	18/02/13	13/06/13	22/10/13
P2	F; 47	10/10/12	13/12/12	27/03/13	31/07/13	13/11/13
P3	F; 49	20/10/10	13/12/10	14/03/11	16/06/11	22/09/11
P4	M; 20	15/10/12	18/12/12	25/02/13	27/03/13	05/11/13

nosis, but based on extracts or proteins they cannot predict the severity of clinical symptoms or the outcome of AIT [1, 2, 5]. Novel approaches such as epitope-based serum diagnostics may improve the identification of clinically relevant allergen-specific antibodies [6, 7]. Although currently not a routine measure, recent evidence suggests a link between IgE epitope traits (e.g., diversity and recognition by different antibody isotypes) with the intensity of peanut and milk allergy [8]. Rapid, inexpensive, and high-throughput methods for the identification of IgE- and IgG-allergen epitopes from patient sera are needed to identify clinically relevant epitopes in large patient cohorts. Current methods such as peptide phage display combined with next generation sequencing [6, 9–13] and overlapping peptide microarrays [7, 8] have severe limitations regarding the small number of identified epitopes or the inability to identify conformational epitopes, respectively. We recently reported the identification of more than 400 potential epitope motifs from soya allergens directly by antibodies from sensitized patients' sera using a peptide phage display platform followed by epitope validation through peptide microarrays [10]. This method is based on a unique statistical motif-oriented analysis approach of peptide phage display data [10, 12]. Here, we aimed to investigate the feasibility of this method for *in silico* identification of natural (seasonal) variations on the recognition of Bet v 1 epitopes and its PR-10 homologs in soya (Gly m 4) and hazelnut (Cor a 1) by serum antibodies in patients sensitized to Gly m 4 collected over 1 year.

Materials and Methods

Patients

Four sera from patients (2 female, mean age 36 years) having participated in the placebo arm of the BASALIT trial (EudraCT-No. 2009-011737-27) [4] were investigated. All patients had a history of birch pollen allergy and were positive for specific IgE against Bet v 1 and Gly m 4 (cutoff: 0.7 kU/L, ImmunoCAP; Ther-

mo Fisher Scientific, Dreieich, Germany). Patients had pollen-related soya allergy diagnosed by positive food challenge with soya. They had no specific IgE against Gly m 5 or Gly m 6. Serum was initially collected before the first placebo injection (t_1) in autumn 2010 (Patient P3) or autumn 2012 (Patient P1, P2, and P4), respectively [4]. Further samples were collected in late autumn (t_2), late winter/spring (t_3), spring/summer (t_4), and autumn (t_5) (Table 1). At each time point, specific IgE concentrations against recombinant Bet v 1, Cor a 1, and Gly m 4 were documented [4].

Peptide-Phage Display

Peptide-phage display experiments were performed using 24 µg of total serum protein immobilized in Immuno™ tubes (Maxi-Sorp™; Thermo Fisher Scientific, Waltham, MA, USA), following an established peptide phage display protocol [10, 12] described in online supplementary Figure E1a (for all online suppl. material, see www.karger.com/doi/10.1159/000509995). The dataset corresponding to t_5 of patient P1 was not included in further analysis since it did not comply with all quality filters. The remaining datasets contained more than 2×10^5 individual unique and enriched sequences. The sequence's frequency is representative for the number of peptide phage bound, specifically and non-specifically, to the immobilized serum proteins/antibodies [10, 12].

In silico Evaluation of Allergen-Related Motifs

Epitope motifs were identified as described [10, 12] and outlined in online supplementary Figure E1a and E1b. For practical reasons, we analyzed all datasets for variations in 4-mer motif statistics (i.e., enrichment values) [10, 12]. The amino acid distribution in the library is essentially even, allowing among others to compare the theoretically expected frequencies of 4-mer motifs with the actual observed values [10]. Motifs with the largest observed variations after the birch pollen season were selected. Additionally, the average number of peptide variants containing a given motif was calculated (i.e., mean of sequences' frequency) for those motifs in which the variation of enrichment values occurred in more than 1 patient.

Results

In silico comparison of peptide datasets obtained from phage display experiments with whole sera reveals that the frequency of certain peptide motifs relates to season-

al variations in birch pollen exposure. Their differential enrichment relates to concentrations of Bet v 1-, Gly m 4-, and Cor a 1-specific antibodies measured in the investigated patient sera. In the selection procedure, no difference is made between different antibody classes; hence, these results reflect the overall increase of allergen-specific immunoglobulins.

Figure 1 shows the individual annual variations in the mean frequency (i.e., the average number of sequences containing a certain motif within a given dataset) of sequences with selected allergen-epitope motifs. These motifs, listed with their amino acid composition and position within their respective allergen primary sequence, are located at the surface of Bet v 1 (online suppl. Fig. E2) and its homologs Cor a 1 and Gly m 4: (1) Bet v 1₃₉ISSV₄₂,₄₀SSVE₄₃ and Cor a 1₃₉ITSV₄₂,₄₀TSVE₄₃, (2) Cor a 1₈₄YTVI₈₇,₈₆VIEG₈₉ and Gly m 4₈₄SVVG₈₈, and (3) Cor a 1₁₅₅HSAE₁₅₉, and Gly m 4₁₅₄HPDY₁₅₇. They were also identified as part of epitopes reported in the Immune Epitope Database (<https://www.iedb.org/>) or epitope candidates identified in-house (online suppl. Table E1).

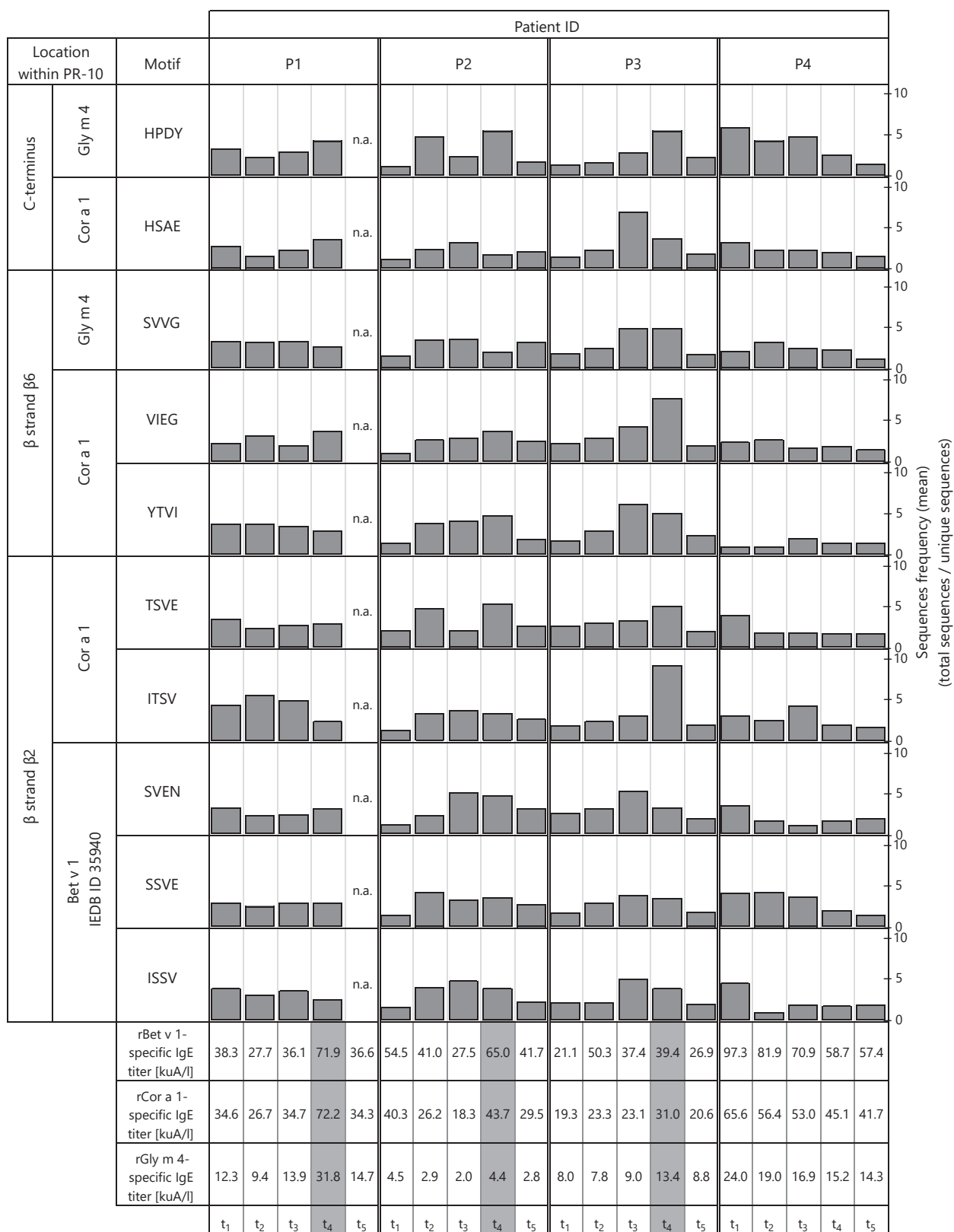
Interestingly, the variations (Fig. 1) match the regional pollen count records (German Weather Service; DWD; <https://www.dwd.de/DE/leistungen/pollen/pollenstatistik.html>) (online suppl. Fig. E3). Patients like P2 and P3 showed larger increases of the frequency of sequences containing distinct motifs as detected by serum antibodies before and after the peak of birch pollen season (t_3 and t_4 , respectively). Potential cross-reactivity of birch- and hazel epitopes is supported by this statistical increase of enriched sequences containing certain Bet v 1- and Cor a 1 motifs in the sera of patients P3 and P4 before the birch pollen season, that is, at time point t_3 (Fig. 1). On the other hand, the highest sIgE concentrations (ImmunoCAP®) (Fig. 1) were reported at time point t_4 in patients P1 and P2, shortly after the beginning of the birch pollen season. Allergen-specific antibody concentrations measured shortly after the peak of the birch pollen season match with our sequencing data and the pollen count records in patients P1 and P2. An example is shown in online supplementary Figure E1b, where after the birch pollen season, a drastic increase in sequences containing a given motif was observed. This increase in the frequency of sequences containing epitope-related motifs can be attributed to elevated antibody concentrations, although it cannot be considered fully quantitative.

Preferences for specific amino acid residues were observed. An example is shown in patient P3 (Fig. 1): at t_4

more sequences containing the motif ITSV (Cor a 1) were observed than for its homolog ISSV (Bet v 1). The number of sequences containing the motif ITSV (Cor a 1) is twice as high as those containing the Bet v 1-motif ISSV (Fig. 2). This would correspond to a primary sensitization to hazel pollen.

Discussion and Conclusion

We investigated variations on the recognition of allergen epitopes by patients' serum antibodies from the BASALIT trial's control group, using samples collected over 1 year. Although during the trial a clear increase in allergen-specific IgG₄ concentration at the end of treatment in verum patients was observed, no significant differences were found on the primary end point (i.e., lowest observed adverse effect level at food challenge) in comparison to the placebo group [4]. This highlights the need of biomarkers to distinguish AIT responders from nonresponders [5]. Methods enabling the identification of personalized epitope profiles might become crucial for predicting severity of allergy, as associations between the intensity of clinical symptoms and the IgE epitope diversity and proximity for milk, peanut, and egg allergy were reported [8, 14]. The establishment of biomarkers to predict AIT outcome is challenging, due to the variety of processes occurring during desensitization, involving not only changes in IgE or IgE-blocking antibody titers [15]. Therefore, IgE binding to allergens and their epitopes alone is not sufficient to predict AIT outcome [15]. There is solid knowledge regarding the epitope-binding profile of IgE and IgE-blocking antibodies elicited during AIT, but limited to only a few patients [16]. Robust methods are needed to study larger numbers of patients, which is essential since every patient has not only a different profile with respect to epitopes but also antibodies targeting the same epitope are from different classes [14, 16]. We used an improved method for in silico analysis of allergen-specific epitope motifs during natural allergen exposure, such as birch pollen. This approach analyzes the seasonal impact on the elicitation of patient antibodies regardless its isotype. For this proof of concept study, 4 patients were sufficient. Larger patient cohorts will be needed to identify season-relevant epitopes because epitope recognition patterns of different patients are highly heterogeneous [7, 9, 10].



1

(For legend see next page.)

Motif Statistics Are Supported by the Measured Concentrations of Allergen-Specific Antibodies and Regional Pollen Counts

Our approach has already been used to identify allergen epitopes of serum antibodies [10, 12]. Because no specific purification step is performed, the identified epi-

tope motifs can be bound and enriched in the experiment by any antibody class. The observed statistical changes must be due to overall changes of moderate and high-affinity binders. The frequency of enriched sequences with allergen epitope motifs therefore correlates with the abundance of epitope-specific serum antibodies. When

Fig. 2. A preference for specific amino acid residues was observed for a hazel-related motif in patient P3 after the birch pollen season (time point t_4). Two subsets of peptide sequences are shown in the alignments: peptides containing the Bet v 1 motif ISSV (above) and the Cor a 1 motif ITSV (below). Sequence identity of the given motif to both allergens is shown with bold, underlined letters. The number and frequency (count) of sequences containing the motif ITSV (35 out of 100,000) doubled the number of sequences containing the motif ISSV (15 out of 100,000). Such difference was not observed when comparing the number of sequences containing these motifs from the naïve library dataset: only 12 sequences contained the motif ISSV (0.5 out of 100,000 sequences) and 17 contained the motif ITSV (0.7 out of 100,000 sequences).

Motif ISSV Patient P3; t ₄	
Protein/Peptide description [Allergen variant, GenBank accession number] [Patient ID Counts]	Protein/peptide sequence
> Bet v 1.0101, CAA33887	NLFPKVAPQA <u>ISSV</u> ENIEGNGGPGT
> Cor a 1.0101, CAA50327	KLIPKVAPQA <u>ITSV</u> ENVEGNGGPGT
> Patient P3 count=5	-----D <u>ISSV</u> YKQHVI AFQLY
> Patient P3 count=8	-----P <u>ISSV</u> YTVFFYYITKC
> Patient P3 count=6	-----DHQCA <u>ISSV</u> NQRPYFC----
> Patient P3 count=4	-----VLVSQR <u>ISSV</u> EQSTHD-----
> Patient P3 count=8	-----QFGCND <u>ISSV</u> YPALYC-----
> Patient P3 count=2	-----GKSCGT <u>ISSV</u> PDLTVY-----
> Patient P3 count=6	-HVMCIDNKKC <u>ISSV</u> CN-----
> Patient P3 count=4	-WFTCHYAHAA <u>ISSV</u> CN-----
> Patient P3 count=7	PRPCFPNAV <u>ISSV</u> Y-----

Motif ITSV Patient P3; t ₄	
Protein/Peptide description [Allergen variant, GenBank accession number] [Patient ID Counts]	Protein/peptide sequence
> Bet v 1.0101, CAA33887	NLFPKVAPQA <u>ITSV</u> ENIEGNGGPGT
> Cor a 1.0101, CAA50327	KLIPKVAPQA <u>ITSV</u> ENVEGNGGPGT
> Patient P3 count=6	-----D <u>ITSV</u> FVNPECFSHIC
> Patient P3 count=19	-----E <u>ITSV</u> ASYRIRPCCIF
> Patient P3 count=20	-----P <u>ITSV</u> DVKEFWDLGGC
> Patient P3 count=8	-----G <u>ITSV</u> HSDHHQNRDQD
> Patient P3 count=15	-----N <u>ITSV</u> SHICGIQVRVF
> Patient P3 count=3	-----D <u>ITSV</u> QFSYCETCLVC
> Patient P3 count=5	-----YWHCE <u>ITSV</u> PESQNFA----
> Patient P3 count=4	-----DQWCTN <u>ITSV</u> RQSDQC-----
> Patient P3 count=10	-----TEMCTE <u>ITSV</u> IHRSSF-----
> Patient P3 count=8	NHVCE <u>IN</u> TR <u>ITSV</u> YI-----
> Patient P3 count=6	ITFSPEHWNE <u>ITSV</u> FD-----
> Patient P3 count=12	YHWSFKEV <u>II</u> <u>ITSV</u> QD-----

Fig. 1. Frequency of allergen-related motifs in NGS data sets and their changes over time in comparison to changes in specific IgE concentrations (ImmunoCAP). Mean of sequence frequencies (i.e., the mean occurrence of unique sequences containing a specific motif in a given peptide dataset; y axis) was calculated for motifs from 3 PR-10 epitopes (left). Bet v 1 epitopes homologous to these epitopes are listed in the IEDB with the following identification numbers: 35,940 (β strand β_2), 67,978 (β strand β_6), and

29,783 (C-terminus). Each dataset corresponds to a serum collected at a defined time point (x axis) in a given patient (top). The highlighting indicates which time point corresponds to serum collected within 6 weeks after the birch pollen season. No time point is highlighted in patient P4 since the only serum taken after the birch pollen season (t_5) was collected in autumn. n.a. dataset not applicable; IEDB, Immune Epitope Database.

comparing Bet v 1-, Gly m 4-, and Cor a 1-specific antibody concentrations with the frequency of sequences containing allergen motifs (Fig. 1), a seasonal increase in the frequency of peptides containing certain allergen motifs was observed in spring/summer. This matches regional pollen count data, but it only partially matches patient specific IgE concentrations (ImmunoCAP). The ImmunoCAP quantifies specific as well as cross-reactive antibodies from a particular class (e.g., IgE) binding the allergen, while our results are influenced by the total Ig for a single (specific) epitope [10]. Neither method is intended to measure individual affinities.

Exposure to Cross-Reactive Allergens Might Influence the Outcome of AIT

Observations from a previous birch-pollen extract-based AIT trial suggested that exposure to other pollen allergens might influence the treatment outcome [17]. In this multinational AIT trial (Finland, Sweden, Poland, Germany), the primary end point reached statistical significance ($p < 0.05$) only when patients from Germany and Southwestern Poland were excluded from the analysis [17]. This effect was attributed to a larger birch pollen concentration and lower interference of other birch-related tree allergens in Scandinavian countries in comparison to Poland and Germany [17]. An influence of seasonal pollen exposure on the success of grass pollen immunotherapy was also reported in trials conducted in Europe and North America [18]. These observations, in addition to our results and its concordance to regional pollen count records, encourage further studies investigating the effects of regional pollen (allergen) exposure during pollen AIT in the individual recognition of related pollen allergens. Our results support the influence of birch and hazel pollen exposure on the induction of cross-reactive antibodies. Patient antibodies for Cor a 1 could be more specific or frequent than those for the homologous motifs in Bet v 1 or Gly m 4 after exposure to birch pollen. This would explain the elicitation of cross-reactive Cor a 1 epitopes, in particular those with the ITSV-motif, as an immune response to birch pollen. Commonly observed symptoms of pollen-related food allergy occurring beyond the birch pollen season have been attributed to such cross-reactivity [1].

Implications for Design and Evaluation of Future Birch AIT

Only data from 4 patients in this study already show that our phage display approach allows characterization of seasonal variations in allergen-specific antibodies at

the epitope level. Future work with more samples will show whether these correlate with the severity of allergic disease. This will also contribute to a better understanding of the impact of seasonal pollen exposure on the treatment success of AIT [18]. Our method allows time-resolved studies, comparing patients' epitope profiles before and after AIT. These results can help determine whether the elicitation of cross-reactive antibodies might influence the outcome of birch pollen AIT and whether a detailed individual epitope profile might drive a more specific and efficient treatment decision [19]. It has to be tested whether peptide epitopes differing by single amino acids could be used in personalized diagnostic methods. This could enable individual *theratype* [19] determination through the application of this method to a larger patient cohort.

Acknowledgements

We highly appreciate the valuable contribution during the mapping of PR-10 allergens of Dorothee Wehrmann, Sandra Haas, and Maria Helm. We also acknowledge Nicolas Delaroque for his support performing next-generation sequencing. Serum samples and patient data from the BASALIT study (EudraCT-No. 2009-011737-27) were kindly provided by the trial's investigators.

Statement of Ethics

Serum samples used in this study were collected from patients recruited for the BASALIT trial (EudraCT-No. 2009-011737-27) after the positive vote from the leading ethics committee located at the University of Leipzig Medical Center had been obtained. All patients provided written informed consent.

Conflict of Interest Statement

M.S. is the CEO and partial owner of Epitopic GmbH, a company that has co-developed crucial software, vectors, and methods described in this manuscript. No direct financial profit has been made from this work. L.R.C., C.K., R.T., J.C.S., U.J., and K.K. have no conflicts of interest to declare.

Funding Sources

The BASALIT trial was funded by the German Federal Ministry of Education and Research (FK 01KG0911). Preliminary results from the project "FoodAllergen," funded by the Fraunhofer Zukunftsstiftung, were included in this manuscript. L.R.C. received funding from this source and the Fraunhofer TALENTA start stipend.

Author Contributions

L.R.C. performed further data analysis and was responsible for drafting the manuscript with input from all authors. C.K. performed all peptide phage display experiments and initial in silico analysis as part of his master thesis. M.S. initiated and supervised

the project. K.K. was responsible for the identification of epitope candidates from the FoodAllergen project. R.T., J.C.S., and U.J. were investigators of the BASALIT trial, helped processing patient samples, allowed facilitated and additional access to patients' serum samples, and supported data analysis.

References

- Biedermann T, Winther L, Till SJ, Panzner P, Knulst A, Valovirta E. Birch pollen allergy in Europe. *Allergy*. 2019;74(7):1237–1248. <http://dx.doi.org/10.1111/all.13758>. 30829410.
- Blankstijn MA, Knulst AC, Knol EF, Le TM, Rockmann H, Otten HG, et al. Sensitization to PR-10 proteins is indicative of distinctive sensitization patterns in adults with a suspected food allergy. *Clin Transl Allergy*. 2017;7:42.
- Skamstrup Hansen K, Vieths S, Vestergaard H, Skov PS, Bindslev-Jensen C, Poulsen LK. Seasonal variation in food allergy to apple. *J Chromatogr B Biomed Sci Appl*. 2001;756(1–2):19–32.
- Treudler R, Franke A, Schmiedeknecht A, Ballmer-Weber B, Worm M, Werfel T, et al. BASALIT trial: double-blind placebo-controlled allergen immunotherapy with rBet v 1-FV in birch-related soya allergy. *Allergy*. 2017;72(8):1243–53.
- Pfaar O, Alvaro M, Cardona V, Hamelmann E, Mösges R, Kleine-Tebbe J. Clinical trials in allergen immunotherapy: current concepts and future needs. *Allergy*. 2018;73(9):1775–83.
- Ehlers AM, Blankstijn MA, Knulst AC, Klinge M, Otten HG. Can alternative epitope mapping approaches increase the impact of B-cell epitopes in food allergy diagnostics?. *Clin Exp Allergy*. 2019;49(1):17–26. <https://dx.doi.org/10.1111/cea.13291>.
- Kühne Y, Reese G, Ballmer-Weber BK, Niggemann B, Hanschmann KM, Vieths S, et al. A novel multi-peptide microarray for the specific and sensitive mapping of linear IgE-binding epitopes of food allergens. *Int Arch Allergy Immunol*. 2015;166(3):213–24.
- Eiwegger T, Hung L, San Diego KE, O'Mahony L, Upton J. Recent developments and highlights in food allergy. *Allergy*. 2019;74(12):2355–67.
- Christiansen A, Kringelum JV, Hansen CS, Bøgh KL, Sullivan E, Patel J, et al. High-throughput sequencing enhanced phage display enables the identification of patient-specific epitope motifs in serum. *Sci Rep*. 2015;5:12913.
- Kern K, Havenith H, Delaroque N, Rautenberger P, Lehmann J, Fischer M, et al. The immunome of soy bean allergy: comprehensive identification and characterization of epitopes. *Clin Exp Allergy*. 2019;49(2):239–51.
- Chen X, Dreskin SC. Application of phage peptide display technology for the study of food allergen epitopes. *Mol Nutr Food Res*. 2017;61(6):1600568.
- Havenith H, Kern K, Rautenberger P, Spiegel H, Szardenings M, Ueberham E, et al. Combination of two epitope identification techniques enables the rational design of soy allergen Gly m 4 mutants. *Biotechnol J*. 2017;12(2):1600441.
- Wu CH, Liu IJ, Lu RM, Wu HC. Advancement and applications of peptide phage display technology in biomedical science. *J Biomed Sci*. 2016;23:8.
- Suprun M, Getts R, Grishina G, Tsuang A, Suárez-Fariñas M, Sampson HA. Ovomucoid epitope-specific repertoire of IgE, IgG4, IgG1, IgA1 and IgD antibodies in egg allergic children. *Allergy*. 2020;. <http://dx.doi.org/10.1111/all.14357>
- Shamji MH, Ljørring C, Würtzen PA. Predictive biomarkers of clinical efficacy of allergen-specific immunotherapy: how to proceed. *Immunotherapy*. 2013;5(3):203–6.
- Gepp B, Lengger N, Möbs C, Pfützner W, Radauer C, Bohle B, et al. Monitoring the epitope recognition profiles of IgE, IgG1, and IgG4 during birch pollen immunotherapy. *J Allergy Clin Immunol*. 2016;137(5):1600–3.e1.
- Worm M, Rak S, Samoliński B, Antila J, Höiby AS, Kruse B, et al. Efficacy and safety of birch pollen allergoid subcutaneous immunotherapy: a 2-year double-blind, placebo-controlled, randomized trial plus 1-year open-label extension. *Clin Exp Allergy*. 2019;49(4):516–25.
- Durham SR, Nelson HS, Nolte H, Bernstein DI, Creticos PS, Li Z, et al. Magnitude of efficacy measurements in grass allergy immunotherapy trials is highly dependent on pollen exposure. *Allergy*. 2014;69(5):617–23.
- Pfaar O, Bonini S, Cardona V, Demoly P, Jakob T, Jutel M, et al. Perspectives in allergen immunotherapy: 2017 and beyond. *Allergy*. 2018;73:5–23.