

Molecular spreading and predictive value of preclinical IgE response to *Phleum pratense* in children with hay fever

Laura Hatzler,^a Valentina Panetta, MSc,^{a,b} Susanne Lau, MD, PhD,^a Petra Wagner,^a Renate L. Bergmann, MD,^c Sabina Illi, PhD,^d Karl E. Bergmann, MD,^e Thomas Keil, MD, MSc,^f Stephanie Hofmaier,^a Alexander Rohrbach,^a Carl Peter Bauer, MD,^g Ute Hoffman, MD,^g Johannes Forster, MD,^h Fred Zepp, MD,ⁱ Antje Schuster, MD,^j Ulrich Wahn, MD,^a and Paolo Maria Matricardi, MD^a Berlin, Munich, Freiburg, Mainz, and Dusseldorf, Germany, and Rome, Italy

Background: IgE sensitization against grass pollen is a cause of seasonal allergic rhinitis.

Objective: We sought to investigate the evolution at the molecular level and the preclinical predictive value of IgE responses against grass pollen.

Methods: The German Multicentre Allergy Study examined a birth cohort born in 1990. A questionnaire was administered yearly, and blood samples were collected at 1, 2, 3, 5, 6, 7, 10, and 13 years of age. Grass pollen-related seasonal allergic rhinitis (SARg) was diagnosed according to nasal symptoms in June/July. Serum IgE antibodies to *Phleum pratense* extract and 8 *P. pratense* molecules were tested with immune-enzymatic singleplex and multiplex assays, respectively.

Results: One hundred seventy-seven of the 820 examined children had SARg. A weak monomolecular/oligomolecular IgE

response to *P. pratense* was observed very frequently before SARg onset. These initial IgE responses increased in concentration and molecular complexity during the preclinical and clinical process. A typical progression of IgE sensitization was observed: Phl p 1 (initiator in >75% of cases); then Phl p 4 and Phl p 5; then Phl p 2, Phl p 6, and Phl p 11; and then Phl p 12 and Phl p 7. At age 3 years, IgE sensitization predicted SARg by age 12 years (positive predictive value, 68% [95% CI, 50% to 82%]; negative predictive value, 84% [95% CI, 80% to 87%]). At this preclinical prediction time, the number of recognized molecules and the serum levels of IgE to *P. pratense* were significantly lower than at 3 or more years after SARg onset. **Conclusions:** The IgE response against grass pollen molecules can start years before disease onset as a weak monosensitization or oligosensitization phenomenon. It can increase in serum concentration and complexity through a “molecular spreading” process during preclinical and early clinical disease stages. Testing IgE sensitization at a preclinical stage facilitates prediction of seasonal allergic rhinitis at its molecular monosensitization or oligosensitization stage. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Allergenic molecules, allergic rhinitis, children, component-resolved diagnosis, component-resolved therapy, grass pollen, hay fever, immunoglobulin E, *Phleum pratense*, Phl p 1, prediction, timothy grass

From the Departments of ^aPaediatric Pneumology and Immunology and ^cPaediatrics and Obstetrics and ^fthe Institute of Social Medicine, Epidemiology and Health Economics, Charité University Medical Centre, Berlin; ^bL'altrastatistica srl, Consultancy and Training, Biostatistics Office, Rome; ^dUniversity Children's Hospital Munich, Department of Pulmonary and Allergy, LMU, Munich; ^ethe Robert Koch Institute, Berlin; ^gthe Department of Pediatrics, Technical University of Munich; ^hSt Josefs Hospital, Department of Pediatrics, Freiburg; ⁱthe Department of Pediatrics and Adolescent Medicine, Johannes Gutenberg University Medical Centre, Mainz; and ^jthe Department of Pediatric Cardiology and Pneumology, Heinrich-Heine-University, Dusseldorf.

Supported by Deutsche Forschungsgemeinschaft (DFG) MA-4740/1-1. The Multicentre Allergy Study cohort was supported by several grants from the German Ministry for Education and Research (Bundesministerium für Bildung und Forschung; reference nos. 07.015.633 ALE27; 01EE9405/5; 01EE9406).

Disclosure of potential conflict of interest: S. Lau has consulted for the Merck Drug monitoring committee; is employed by Charité; has received grants from the German Research Foundation, SymbioPharm, and Airsonett; and has received payment for lectures from AstraZeneca, Novartis, and SymbioPharm. K. E. Bergmann has received grants from and is employed by the Federal Office of Health in Berlin, Germany; has consulted for the European Union; and has received payment for lectures and development of education presentations from AOK Baden. T. Keil has received grants from the German Research Foundation. F. Zepp is a member of the board of Glaxo-SmithKline, Novartis, Pfizer, and Sanofi Pasteur; has consulted for Novartis and GlaxoSmithKline; has received payment for lectures from GlaxoSmithKline and Novartis; and has received payment for development of educational presentations from Pfizer. A. Schuster has received grants from the German Ministry for Education and Research and has received payment for lectures from Thermo Fisher. U. Wahn has received grants from the German Ministry of Research and Education. P. M. Matricardi has received grants from the Deutsche Forschung Gesellschaft (DFG) and Thermo Fisher Scientific, has consultant arrangements with Allergopharma, and has received payment for lectures from Thermo-Fisher Scientific. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 20, 2012; revised May 24, 2012; accepted for publication May 30, 2012.

Corresponding author: Paolo Maria Matricardi, MD, Department of Paediatric Pneumology and Immunology, Charité Medical University, Augustenburger Platz, 1, 13353 Berlin, Germany. E-mail: paolo.matricardi@charite.de.

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology
<http://dx.doi.org/10.1016/j.jaci.2012.05.053>

Allergic sensitization to grass pollen and hay fever are highly frequent among adults^{1,2} and children³ in developed countries, and their burden is enormous.⁴ Avoidance of grass pollen allergens is difficult, drug treatment is only partially effective, and no cure is available.⁵ Allergen-specific immunotherapy (SIT) is effective in the short-term^{6,7} and long-term,⁸ but its efficacy is partial⁹ and debated.¹⁰

The diagnostic and therapeutic approach to grass pollen allergy is mainly based on grass pollen extract preparations. However, extracts produced from different companies are heterogeneous in their molecular composition.¹¹ On the other hand, the IgE sensitization profiles of patients with established clinically relevant allergic sensitization to grass pollen are also very heterogeneous.¹² Mismatch in the molecular sensitization profile of an individual patient and the molecular profile of the allergenic preparation might explain a reduced diagnostic and therapeutic performance.¹²

Our understanding of IgE-mediated allergies has greatly improved since the advent of “molecular allergology.”¹³ Under this approach, molecules, instead of extracts, are used for both the diagnosis (component-resolved diagnosis) and therapy (component-resolved therapy) of allergic diseases.¹⁴ This concept

Abbreviations used

ISAC: Immuno Solid-phase Allergen Chip
 ISU: ISAC Standard Unit
 MAS: Multicentre Allergy Study
 SARg: Grass pollen-related seasonal allergic rhinitis
 SIT: Allergen-specific immunotherapy

foresees characterizing the patient's sensitization profile at the molecular level and, with this information, tailoring the composition of his or her individualized SIT.¹⁴

At least 13 allergenic molecules of *Phleum pratense* (timothy grass), a representative species of the Poaceae family, have been identified and sequenced,¹⁵ and the prevalence of an IgE response to 8 of them has been repeatedly examined in children and adults.¹⁶ IgE sensitization molecular profiles of children with established disease are complex and heterogeneous,¹² so that tailoring component-resolved therapy at advanced disease stages might be difficult.¹⁴ However, the origins and molecular evolution of IgE responses against grass pollen have never been investigated. Therefore whether IgE sensitization is less complex and heterogeneous at earlier stages of the disease process is presently unknown.

To answer this question, we have taken advantage of the Multicentre Allergy Study (MAS), a birth cohort study starting in 1990. The MAS has scheduled yearly evaluation of upper airway symptoms and repeated peripheral blood drawing at 8 follow-up points during the first 13 years of life.¹⁷ We could therefore retest the sera of participants with a molecular approach and could match this molecular analysis with the clinical history of seasonal allergic rhinitis.

METHODS

Study cohort

The MAS, a prospective observational birth cohort study, recruited 1314 of 7609 infants born in 1990 on 6 delivery wards in 5 German cities (Berlin, Dusseldorf, Mainz, Freiburg, and Munich). A detailed description of the stratified sampling scheme and study subjects is given elsewhere¹⁷ and in the **Methods** section in this article's Online Repository at www.jacionline.org. The study was approved by the local ethics committee. Each parent provided written informed consent at the time of enrollment. Blood samples were collected at 1, 2, 3, 5, 6, 7, 10, and 13 years of age.

Definitions

A standardized parental questionnaire/interview was used yearly (including International Study of Allergy and Asthma in Childhood questions) to assess allergic symptoms. We examined symptoms of allergic rhinitis related to grass pollen, as defined by the presence of "reported sneeze attacks or a runny, blocked, or itchy nose in the absence of common cold"¹⁸ in the months of June, July, or both preceding the follow-up visit. A subject was considered to be affected by grass pollen-related seasonal allergic rhinitis (SARg) if symptoms were reported in 3 or more follow-ups between 3 and 13 years of age or at least 2 of the 3 follow-ups between 11 and 13 years of age. The age at onset of SARg was defined as the age at the first follow-up in which symptoms of SARg had been reported.

IgE assays

All the available serum samples were tested for IgE antibodies against the extracts of *P pratense* with the ImmunoCAP Fluorescence Enzyme Immuno-Assay (Thermo Fisher Scientific, Uppsala, Sweden). Results

TABLE I. Characteristics of the population

Variable	Study population	Excluded	P value*
No. of subjects	820	494	
Male sex (%)	427/820 (52.1)	257/494 (52.0)	.99
Parental history of allergy (%)	443/816 (54.3)	237/487 (48.7)	<.05
German nationality	764/800 (95.5)	435/469 (92.8)	.04
Older siblings	344/820 (42.0)	194/494 (39.7)	.48
Parental education (≥ 12 y [%])	445/794 (56.0)	218/478 (45.6)	<.001
Breast-feeding (up to 6 mo)	622/813 (76.5)	271/479 (56.6)	<.001
Mother smoking (at child's age of 5 y)	274/820 (33.4)	141/285 (49.5)	<.001
SARg	177/820 (21.6)	NA	
Age at onset (y), median (interquartile range)	7 (5-9)		
Incidence rate (%) [95% CI]	2.3 (1.9-2.6)		

NA, Not applicable.

*P values are for comparison between subjects of the MAS cohort included and excluded from the study (χ^2 test).

were expressed in kilounits per liter (detection range, 0.35-100 kU_A/L). Sera with a concentration of specific IgE antibodies greater than 80% of the upper detection limit of the assay were diluted 1:5 to obtain a precise determination. A result of 0.35 kU_A/L or greater was considered positive. Sera with positive results to the extract of *P pratense* were tested again, if still available, with a microarray assay (Immuno Solid-phase Allergen Chip [ISAC]; Thermo Fisher Scientific, Vienna, Austria) to characterize the molecules recognized by their IgE antibodies. The details of the methods are reported elsewhere and in the **Methods** section in this article's Online Repository.¹⁹ In its version containing 103 molecules, each one arrayed in triplicates, the ISAC test includes 8 molecules of *P pratense* (rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5 b, rPhl p 6, rPhl p 7, rPhl p 11, and rPhl p 12). A result of low, middle, or high positivity was considered positive.

Statistics

The data were analyzed by 2 of us (P.M.M. and V.P.). The data from sera obtained after the initiation of grass pollen SIT (see also the **Methods** section in this article's Online Repository) were excluded from any statistical analysis. The Shapiro-Wilk test was used to evaluate normal distribution of quantitative variables. The average concentration of IgE to *P pratense* extract in positive sera was calculated as the geometric mean value. Comparisons between participants included and excluded from the analysis were examined by using χ^2 tests. Frequencies for SARg and each *P pratense* molecule were calculated. In survival analyses patients were censored if they had not experienced the end point of interest at the end of the follow-up period. Kaplan-Meier estimates of overall survival time were compared by using the log-rank test; 25% SARg-free time was used as one of our ranking systems of *P pratense* molecular order. Two additional ranking system orders were based on average yearly incidence and on the appearance order of each molecule. Because the length of the follow-up period differed among the patients, all analyses taken into account comprised 5 years before and 5 years after the onset at maximum. Multilevel models were used for multiple values of the same patients during the follow-up. Multilevel mixed-effects linear regression (xtmixed command) and multilevel mixed-effects Poisson regression (xtmepoisson command) were used to evaluate the effects of delay from onset corrected for age at onset on logarithmic transformation of IgE to *P pratense* and the number of *P pratense* molecules, respectively. Separately, models for relationships before and after onset were provided. Multilevel mixed-effects logistic regression was used to evaluate the risk of SARg in the next 3 years for IgE-positive subjects compared with IgE-negative subjects corrected by age. Three different models were provided to evaluate other possible confounding or risk factors. For all multilevel analysis, random effects are estimated by using a multiple of identity matrix (identity option).

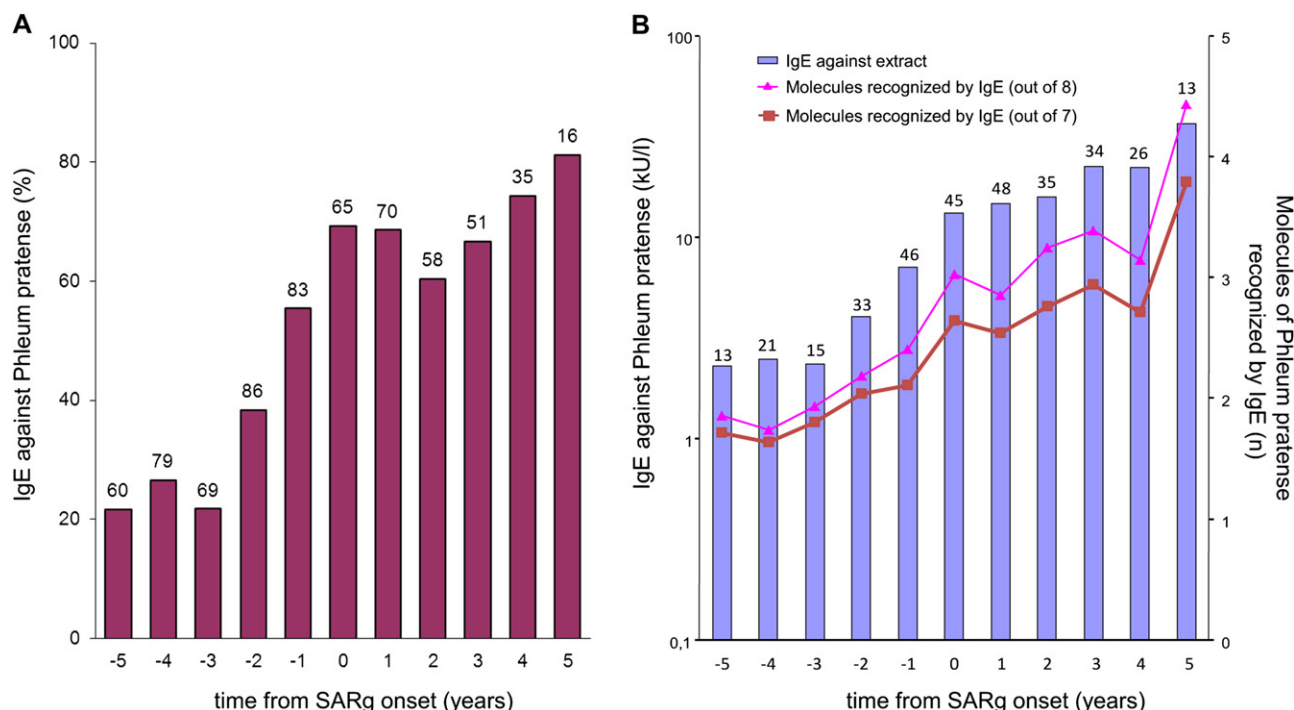


FIG 1. IgE to *P pratense* by time from onset of grass-related seasonal allergic rhinitis. **A**, Bars show the prevalence of IgE sensitization (≥ 0.35 kU_A/L) to *P pratense* (extract) in children whose sera were available at each point in time. The number of tested children is indicated over each bar. **B**, Bars show the geometric mean levels of IgE antibodies to *P pratense* (extract) at each point in time in IgE antibody-positive sera. The numbers of tested children is indicated over each bar. Lines show the average number of all 8 (triangles) or only 7 (squares; Phl p 6 excluded) allergenic molecules of *P pratense* recognized by IgE antibodies at each point in time in IgE antibody-positive sera.

Odds ratios and their 95% CIs were calculated, and relevant confounding variables were included in the final model. Two-by-two tables were used to calculate the sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios. Comparisons between IgE values obtained at preclinical versus clinical follow-up points from the same children with SARg have been performed by using χ^2 tests or exact tests for dichotomous variables and *t* tests for paired-sample or Wilcoxon matched-pairs tests for quantitative variables. Statistical analysis was performed with Stata 12.0 software (StataCorp, College Station, Tex). A *P* value of less than .05 was considered statistically significant.

RESULTS

Study cohort

The study cohort consisted of 820 subjects (427 male and 393 female subjects) of the initial 1314 participants of the MAS birth cohort (Table I). German nationality, higher parental education, longer breast-feeding duration, a nonsmoking mother, and parental history of allergy were found more frequently in children included than in those excluded from the study (Table I). SARg has been identified in 177 (21.6%) of the 820 participants. The percentage of new cases with SARg increased at a constant rate of 2.3% per year between the ages of 3 and 12 years (see Fig E1 in this article's Online Repository at www.jacionline.org), its average incidence rate was 2.3 (95% CI, 1.9% to 2.6%), and the median age at onset was 7 years (interquartile range, 5-9 years). Details on the participation rates to follow-up interviews and blood draws and the availability of IgE tests and on reported SIT are presented in the Results section and Table E1 in this article's Online Repository at www.jacionline.org. The present

study is based on the analysis of 4173 sera obtained from 820 children, corresponding to 65.7% of their scheduled blood samples.

Progressive IgE response to *P pratense*

The prevalence of positive test results for serum IgE antibodies to the timothy pollen extract in children with SARg increased with the time from disease onset (Fig 1, A). The geometric mean concentration of IgE antibodies to timothy pollen in positive sera also increased with the time from disease onset; average values were less than 4.0 kU_A/L (95% CI, 3.0-5.4 kU_A/L) in the preclinical phase, increased to 13.1 kU_A/L (95% CI, 7.8-22.3 kU_A/L) at disease onset, increased to 15.0 kU_A/L (95% CI, 10.2-22.1 kU_A/L) in the 2 years after disease onset, and peaked at 24.3 kU_A/L (95% CI, 16.0-36.9 kU_A/L) in sera collected at least 3 years after disease onset (Fig 1, B). Multilevel mixed-effects linear regression showed a significant increment in the mean IgE antibody to timothy pollen value considering time at onset (after adjusting for age at onset), with an IgE geometric mean value increase of 1.5 kU_A/L (95% CI, 1.4-1.6 kU_A/L; *P* < .001) each year. An average increment of 1.8 kU_A/L (95% CI, 1.6-2.1 kU_A/L; *P* < .001) was found in the preclinical phase, and an average increment of 1.3 kU_A/L (95% CI, 1.2-1.5 kU_A/L; *P* < .001) was found in the clinical phase. The frequency of subjects with SARg symptoms in the absence of specific IgE is shown in the Fig 1, A. The same panel shows that this percentage is progressively reduced after onset of disease. Hence most seronegative children with SARg become seropositive after onset.

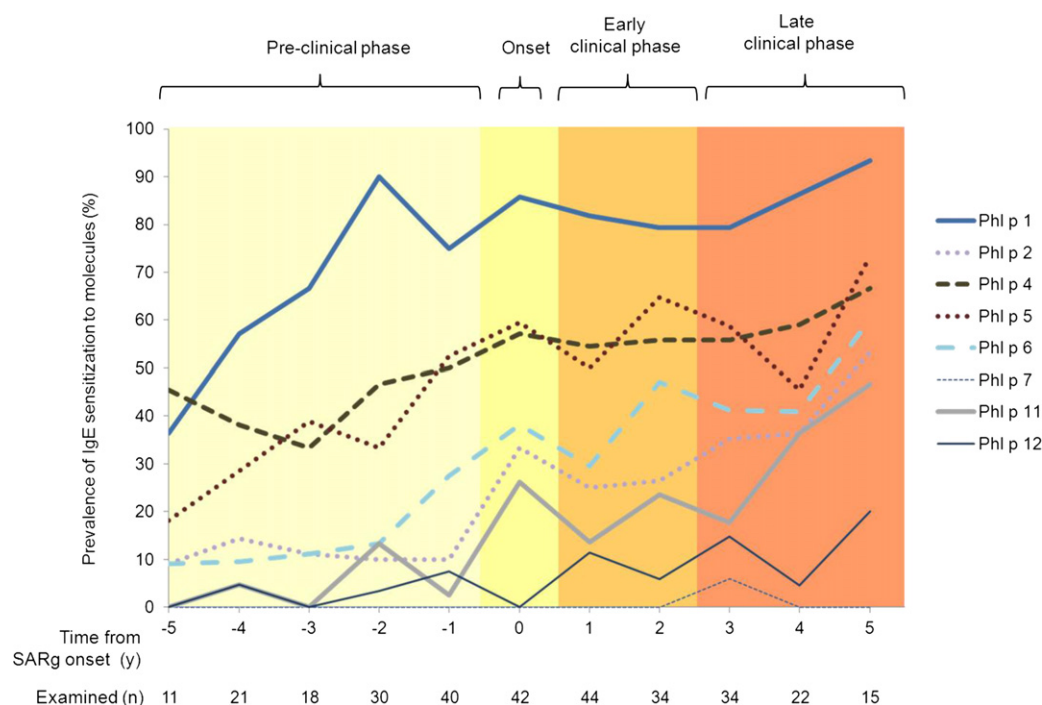


FIG 2. IgE to *P pratense* allergenic molecules from the onset of grass-related seasonal allergic rhinitis. Lines show the prevalence of IgE sensitization (ISAC class ≥ 1) to the 8 *P pratense* allergenic molecules in children whose sera were available at each time point. The number of children examined at each time point is indicated under the x-axis. Clinical stages of seasonal allergic rhinitis are also indicated.

The average number of *P pratense* allergenic molecules recognized by IgE antibodies in positive sera also increased with time from disease onset; average values were 2.1 (95% CI, 1.9-2.4) in the preclinical phase, 3.0 (95% CI, 2.5-3.5) at onset, 3.0 (95% CI, 2.6-3.4) in the 2 years after disease onset, and 3.3 (95% CI, 2.8-3.8) in sera collected at least 3 years after disease onset (Fig 1, B). Multilevel mixed-effects Poisson regression showed a significant increment in molecular numbers, considering time at onset (after adjusting for age at onset). The β regression coefficient was 0.10 (95% CI, 0.06-0.13; $P < .001$), which means an average increment of 0.10 in number of components recognized each year from onset. This association persisted (β regression coefficient, 0.08; 95% CI, 0.05-0.12; $P < .001$) when IgE antibodies against Phl p 6 (sharing B-cell epitopes with Phl p 5²⁰) were excluded from analysis and after adjusting for the concentration of IgE against *P pratense* extract. The average increment in the number of components recognized per year was 0.13 (95% CI, 0.05-0.21; $P = .002$) in the preclinical phase and 0.07 (95% CI, 0.01-0.12; $P = .017$) in the postclinical phase.

Sequential IgE sensitization to *P pratense* molecules

The examined between-patient progression of IgE sensitization to individual *P pratense* molecules is shown in Fig 2. Phl p 1 was the first molecule recognized during the IgE response against timothy pollen in more than 75% of the tested patients. The prevalence of IgE sensitization to the other molecules increased with time but with a slower trend when compared with Phl p 1. Phl p 1, Phl p 5, and Phl p 4 were the molecules most frequently recognized in the preclinical sensitization phase. Phl p 2 and Phl p 6

were recognized only in a minority (14/42 [33.2%] and 16/42 [38.1%], respectively) of the children examined at disease onset and were more frequently recognized only in the postonset years. Phl p 11 and Phl p 12 were recognized mainly in the late postclinical phase. Phl p 7 was recognized exclusively in a few children during the late clinical phase (Fig 2). The analysis of the within-patient individualized sensitization progression performed with 3 different approaches (scoring system, hierarchic incidence, and Kaplan-Meier analysis) confirmed the outcomes obtained on the between-patient progression of IgE sensitization: as a general trend, the first recognized molecule was Phl p 1, followed by Phl p 4 and Phl p 5, then by Phl p 2 and Phl p 6, and then by Phl p 11, Phl p 12, and Phl p 7 (Fig 3 and Table II).

Predictive value and molecular profile of preclinical IgE responses

Among the 643 children without reported SARg, 167 (26%) were sensitized to grass pollen at 1 or more follow-up points in the observation period. An IgE response to grass pollen at 3 years of age was observed in 38 (7.9%) of 482 children, but only 1 of them had already reported SARg. Interestingly, among the remaining 37 children, 25 (67.6%) had SARg at a later follow-up point. The SARg-free curve in 3-year-old children was decreasing among the children with IgE against timothy pollen faster than among the seronegative children ($P < .001$, see Fig E2 in this article's Online Repository at www.jacionline.org). A similar result was obtained when age 6 years was chosen as the prediction time (see Fig E3 in this article's Online Repository at www.jacionline.org). In a multilevel model IgE sensitization to grass pollen (adjusted for age) was strongly associated with the development of SARg, even after

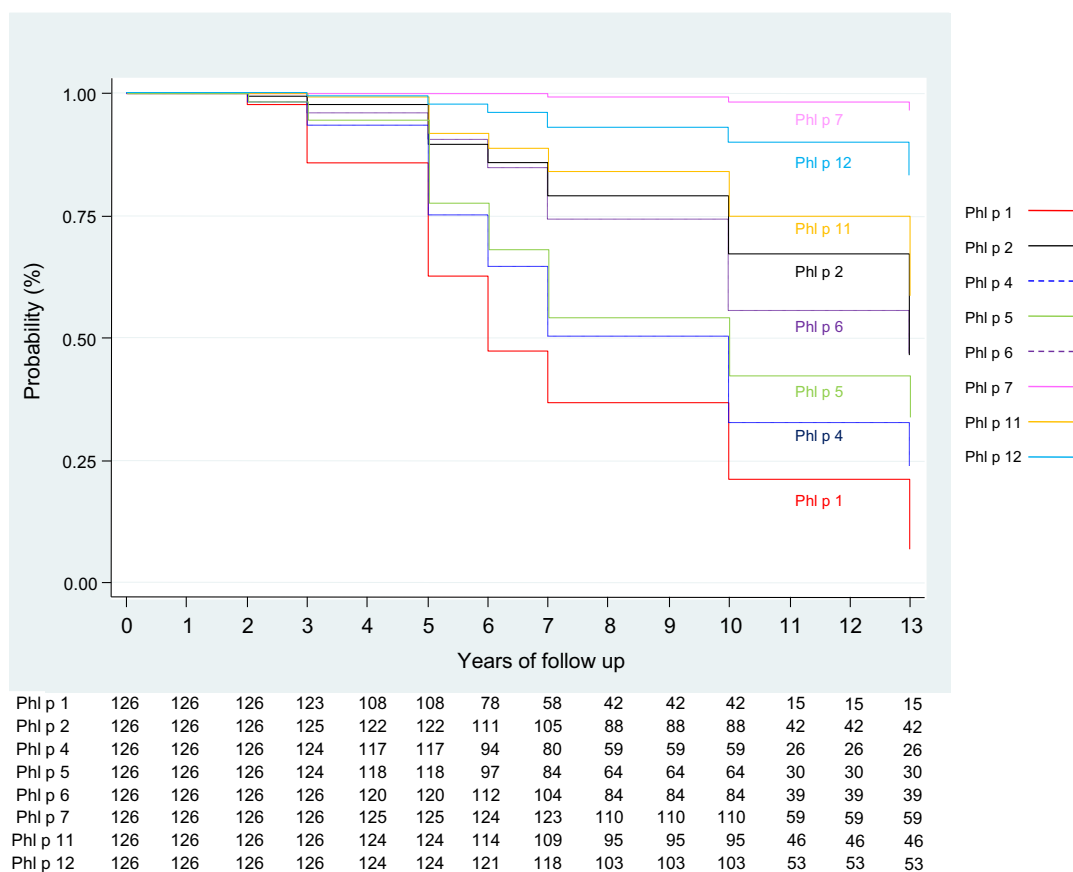


FIG 3. Probability of remaining free of sensitization to individual allergenic molecules of *P pratense*. Kaplan-Meier plots of the probability of remaining free of IgE sensitization to each of 8 allergenic molecules of *P pratense* are shown. The number of the subjects at risk is shown below the x-axis.

TABLE II. Sequence of sensitization to *P pratense* molecules in 126 children* with seasonal allergic rhinoconjunctivitis related to grass pollen allergy

Sequence	Molecules	Positive		Scoring system†			Average yearly incidence (%)	25% Survival time‡ (y)
		No.	Percent	Mean	SD	95% CI		
1	Phl p 1	107	84.9	1.2	0.5	1.1-1.3	11.9	5
2	Phl p 4	93	73.8	1.6	1.0	1.4-1.8	8.6	6
3	Phl p 5	77	61.1	1.7	1.1	1.5-2.0	7.2	6
4/5	Phl p 2	50	39.7	2.3	1.4	1.9-2.7	4.2	10
4/5	Phl p 6	58	46.0	2.8	1.6	2.4-3.2	4.9	7
6	Phl p 11	41	32.5	3.1	2.0	2.5-3.7	3.1	13
7	Phl p 12	12	9.5	4.3	2.1	3.1-5.5	1.2	—
8	Phl p 7	3	2.4	4.7	3.2	1.0-8.3	0.2	—

*Data from 126 of the 177 children with SARg have been analyzed. The remaining 51 cases were not examined because their sera were either missing or negative for IgE antibodies to *P pratense* extract.

†The ranking system was based on the average score in temporal sequence in which each *P pratense* molecule-specific IgE antibody is detected.

‡The ranking system was based on Kaplan-Meier curves (see Fig E2).

adjusting for a list of relevant confounding factors; in this model age was inversely associated with the disease (Table III). Predictive value was very high for nonsensitized children to remain SARg free (negative predictive value, 84%; 95% CI, 80% to 87%). Approximately two thirds (68%; 95% CI, 50% to 82%) of the 3-year-old children sensitized to grass pollen had SARg by age 12 years (Table IV).

The molecular profiles of preclinical IgE sensitization to grass pollen were examined at the chosen prediction times and

compared with those obtained in the same children 3 or more years after SARg onset. The concentration, number of molecules recognized, and heterogeneity of molecular sensitization profiles were all significantly lower at the prediction point than in the clinical phase; one third of the children were monosensitized to Phl p 1 at the prediction times (3 or 6 years of age), but none of them was monosensitized 3 or more years after disease onset (Table V). In this subgroup the average increase per year in the number of *P pratense* molecules recognized by IgE was 0.36 (SD, 0.27).

TABLE III. Relation of IgE against grass pollen and future grass pollen-related seasonal allergic rhinoconjunctivitis

Variable	OR	95% CI	P value
First model*			
Age	0.87	0.8-1.0	.005
IgE grass positive	13.60	8.1-23.0	<.001
Second model†			
Age	0.88	0.8-1.0	.01
IgE grass positive	11.46	6.8-19.4	<.001
Parental history of allergy	3.27	1.8-5.9	<.001
Older siblings	0.68	0.4-1.2	.15

OR, Odds ratio.

*Two-level logistic model: 2422 observations (level 1) for 756 subjects (level 2).

Subjects were in the model up to SARg development. The dependent variable was development of SARg in the next 3 years (binary variable). Independent variables were age (continuous variable) and IgE grass (binary variable).

†Two-level logistic model: 2411 observations (level 1) for 756 subjects (level 2).

Subjects were in the model up to SARg development. The dependent variable was development of SARg in the next 3 years (binary variable). Independent variables were age (continuous variable), IgE grass (binary variable), parental history of allergy (binary variable), and older sibling (binary variable). Additional confounders (sex, parental education, breast-feeding, mother smoking, and German nationality) have not been included in the model because they had *P* values of .10 or greater in univariate analysis.

DISCUSSION

In this birth cohort study we investigated the evolution of IgE sensitization to *P pratense* allergenic molecules in children affected by seasonal allergic rhinitis caused by grass pollen. We found that the IgE response to grass pollen (1) can start years before the perception of the first symptoms and can predict future disease onset, (2) increases in serum concentration before and during the disease process, and (3) is often initiated by Phl p 1 monosensitization and becomes molecularly more and more complex with time.

Previous studies have shown that skin test sensitization to pollen allergens can precede the onset of seasonal allergic rhinitis in childhood and that this information can be used in combination with other risk factors to predict disease onset.^{21,22} This study confirms those observations and additionally shows that the simple detection of preclinical sensitization to grass pollen can allow prediction of the onset of hay fever in an allergen-specific manner. In this case IgE sensitization is indeed not only a risk factor but also a specific disease cause. The results suggest that a precise phenotypic definition of the causative pollen makes hay fever predictable through allergen-specific IgE tests.

Disease onset occurred in many children of the MAS cohort in the middle of a complex immunologic process that started years before disease onset and fully matured only years afterward. Hence the concentration of IgE in the first season of disease was in most patients higher than that observed in a preclinical sensitization stage, and it tended to be lower than that measured 5 years after the disease onset. Similarly, the number of *P pratense* molecules recognized by IgE also increased with time. Taking the 8 molecules of *P pratense* as a reference, many children started as monosensitized (IgE recognition of 1 molecule only), then became oligosensitized (2-3 molecules), and finally became polysensitized (>3 molecules). Interestingly, Phl p 1 was an initiator of sensitization in greater than 75% of the patients. Soon, most patients produced IgE also to Phl p 5 and/or Phl p 4 and then to Phl p 2 and/or Phl p 6 and/or Phl p 11, whereas only some patients produced IgE responses against Phl p 12 and Phl p 7. This sequence reproduces the

prevalence hierarchy already observed in many cross-sectional studies quite well.^{16,23} In analogy with the epitope-spreading process, this phenomenon could be defined as the molecular spreading of the IgE response to an allergenic source. This “molecular spreading” can account for part of the progressive increase in serum IgE antibody levels against *P pratense*, and it is steeper in the preclinical than the clinical stages of the IgE immune response. It could be argued that this phenomenon is an artifact because of cross-reactivity between different components sharing common B-cell epitopes. It is well known that Phl p 6 cross-reacts with Phl p 5.²⁰ In contrast, Phl p 2, notwithstanding a certain degree of sequence homology to Phl p 1 (40% identity and 60% to 70% similarity) does not cross-react with that component.²⁴ In any case, the molecular spreading phenomenon clearly persisted after exclusion of Phl p 6 from the analyses (Fig 1, B, and Table V) and Phl p 6 plus Phl p 2 (data not shown).

Grass pollen-related allergic rhinitis can be hardly cured by SIT, but symptoms can be decreased to approximately 50% to 70%.²⁵ However, usually SIT is advised in patients with a relatively long-lasting history of disease.⁵ Our results show that patients' IgE sensitization is molecularly simpler at its preclinical and early clinical stages than at a time when they usually start receiving SIT. Although the observational nature of our study precludes making clinical recommendations, we hypothesize that SIT would be immunologically and probably also clinically more effective if started earlier during the disease process. In addition, an allergen-specific immune intervention might even prevent disease or delay its onset if started in the preclinical sensitization phase (ie, specific immunoprophylaxis). However, one should caution about the alternative although unlikely hypothesis that an allergen-specific immune intervention in the preclinical sensitization phase might accelerate rather than prevent symptom onset. Early immunologic intervention in molecularly oligosensitized subjects was theorized about a decade ago.¹⁴ The present results provide a solid background to future trials testing that seminal hypothesis and suggest also that a molecular approach to early immune intervention (component-resolved prophylaxis and earlier component-resolved therapy) would be, given the lower number of molecules involved, more feasible.

Some limitations of our study should be considered. First, we examined only children living in Germany, in whom grass and birch pollen allergies are responsible for the vast majority of seasonal allergic rhinitis. We caution about generalizing our conclusions to adults and to countries (eg, Mediterranean countries) with different climates and many other relevant allergenic pollen sources.^{26,27}

Second, we have examined only one birth cohort, and a relatively consistent proportion of missing blood samples might have generated a participation bias. The baseline characteristics were found to be different between the subsample and the study population in terms of family history for atopy, smoking, and breast-feeding, meaning that atopic parents were probably more likely to continue participation and to avoid known risk factors. Although this might have influenced the likelihood of sensitization, this is not necessarily true for the development and molecular spreading of sensitization. However, no other large birth cohort study on allergies, to our knowledge, has collected clinical and serologic information at so many points in time (ie, from birth to adolescence). Therefore the evaluation of a second birth cohort study with the same characteristics of the MAS might be at present impossible.

TABLE IV. Prediction of SARg based on IgE levels against grass pollen (>0.35 kU_A/L)

	IgE+		IgE–		Sensitivity (% [95% CI])	Specificity (% [95% CI])	PPV (% [95%CI])	NPV (% [95% CI])	LR+	LR–
	SARg positive (no.)	SARg negative (no.)	SARg positive (no.)	SARg negative (no.)						
At age 3 y, limited to the following 3 y	18	19	25	414	41.9 (27.1-57.9)	95.6 (93.2-97.3)	48.6 (31.9-65.6)	94.3 (91.7-96.3)	9.5	0.6
At age 3 y, extended up to age 12 y	25	12	72	367	25.8 (17.4-35.7)	96.8 (94.5-98.4)	67.6 (50.2-82.0)	83.6 (79.8-86.9)	8.1	0.8
At age 6 y, limited to the following 3 y	20	50	11	321	64.5 (45.4-80.8)	86.5 (82.6-89.8)	28.6 (18.4-40.6)	96.7 (94.1-98.3)	4.8	0.4
At age 6 y, extended up to age 12 y	28	42	24	308	53.8 (39.5-67.8)	88.0 (84.1-91.2)	40.0 (28.5-52.4)	92.8 (89.4-95.3)	4.5	0.5

LR–, Negative likelihood ratio; LR+, positive likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

TABLE V. Characteristics of the IgE response against *P pratense* in children at the preclinical prediction point (age 3 or 6 years) and at 3 or more years after SARg onset

	No.	At age 3 y	≥3 y After SARg onset	P value	No.	At age 6 y	≥3 y After SARg onset	P value
Concentration of IgE against <i>P pratense</i> extract (kU _A /L), geometric mean (SD)	20	2.5 (5.0)	44.7 (4.1)	<.001	14	6.1 (4.5)	30.0 (2.7)	.005
Molecular spreading (based on Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 11, and Phl p 12)								
Phl p molecules recognized by IgE (no.), median (interquartile range)	15	1 (1-3)	4 (3-6)	<.001	10	1.5 (1-2)	4 (2.8-5)	.005
Subjects with ≥3 molecules (no. [%])	15	6 (40)	15 (100)	.001	10	1 (10)	8 (80)	.005
Phl p 1 only (no. [%])	15	5 (33)	0 (0)	.042	10	4 (40)	0 (0)	.087
Molecular spreading (based on Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 7, Phl p 11, and Phl p 12)								
Phl p molecules recognized by IgE (no.), median (interquartile range)	15	1 (1-3)	4 (3-5)	<.001	10	1.5 (1-2)	3.5 (2-4)	.005
Subjects with ≥3 molecules (no. [%])	15	4 (27)	13 (87)	.001	10	1 (10)	7 (70)	.020
Phl p 1 only (no. [%])	15	5 (33)	0 (0)	.042	10	4 (40)	0 (0)	.087

Third, some of the MAS sera were tested as much as 20 years after their collection, and the results might have been biased by serum deterioration. Nevertheless, all our quality control tests have demonstrated that the IgE antibodies have been preserved very well during this long period (data not shown).

Fourth, *P pratense* does not represent the whole set of allergenic sources of the Poaceae family.^{28,29} However, it has been shown that more than 90% of the IgE sensitization against grass pollen can be measured by using this representative allergenic source.³⁰

Fifth, some of the components of *P pratense* (eg, Phl p 4) might be less clinically relevant than others, although additional clinical data have been claimed to reach a firm conclusion on this point.³¹ On the other hand, sensitization to Phl p 7 (polcalcin) and Phl p 12 (profilin) might have been induced by other pollen sources carrying cross-reactive molecules.³² However, sensitization to Phl p 12 or Phl p 7 was, in the MAS cohort children, almost invariably associated with primary IgE sensitization to Phl p 1, Phl p 5, or both.

In summary, this study showed that a weak IgE response against grass pollen can start years before the perception of the first symptoms, when only a few molecules are recognized. The results also demonstrate that the IgE response becomes progressively stronger and molecularly more and more complex. Finally, the study proves that testing IgE sensitization at a

preclinical stage allows prediction of seasonal allergic rhinitis at its molecular monosensitization or oligosensitization stage.

We thank the study participants and their parents. We also thank Andreas Reich for data management of the general MAS databank, Linus Grabenherr for his contribution to the data collection on SIT in the MAS cohort, and Gabriele Schulz for management of the general MAS sera bank and for the tests of IgE against pollen extracts. We thank Tamara Pace-Ross for English editing.

Clinical implications: Testing IgE sensitization at a preclinical stage facilitates prediction of seasonal allergic rhinitis at its molecular monosensitization or oligosensitization stage.

REFERENCES

1. Burney P, Malmberg E, Chinn S, Jarvis D, Luczynska C, Lai E. The distribution of total and specific serum IgE in the European Community Respiratory Health Survey. *J Allergy Clin Immunol* 1997;99:314-22.
2. Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J* 2004;24:758-64.
3. Strachan D, Sibbald B, Weiland S, Ait-Khaled N, Anabwani G, Anderson HR, et al. Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in childhood. *Pediatr Allergy Immunol* 1997;8:161-76.
4. Nathan R. The burden of allergic rhinitis. *Allergy Asthma Proc* 2007;28:3-9.

5. Bousquet J, Schünemann HJ, Zuberbier T, Bachert C, Baena-Cagnani CE, Bousquet PJ, et al. Development and implementation of guidelines in allergic rhinitis: an ARIA-GA2LEN paper. *Allergy* 2010;65:1212-21.
6. Frew AJ, Powell RJ, Corrigan CJ, Durham SR. UK Immunotherapy Study Group. Efficacy and safety of specific immunotherapy with SQ allergen extract in treatment-resistant seasonal allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 2006;117:319-25.
7. Matricardi PM, Panetta V, Kuna P, Wahn U, Narkus A. Specific immunotherapy and pharmacotherapy in the treatment of seasonal allergic rhinoconjunctivitis: a comparison based on meta-analyses. *J Allergy Clin Immunol* 2011;128:791-9, e6.
8. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999;341:468-75.
9. Plaut M, Valentine MD. Clinical practice. Allergic rhinitis. *N Engl J Med* 2005;353:1934-44.
10. Adkinson NF Jr, Eggleston PA, Eney D, Goldstein DO, Schuberth KC, Bacon JR, et al. A controlled trial of immunotherapy for asthma in allergic children. *N Engl J Med* 1997;336:324-31.
11. Focke M, Marth K, Flicker S, Valenta R. Heterogeneity of commercial timothy grass pollen extracts. *Clin Exp Allergy* 2008;38:1400-8.
12. Tripodi S, Frediani T, Lucarelli S, Macri F, Pingitore G, Di Rienzo Businco A, et al. Molecular profiles of IgE to *Phleum pratense* in children with grass pollen allergy: Implications for specific immunotherapy. *J Allergy Clin Immunol* 2011;129:834-9, e8.
13. Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Grönlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999;29:896-904.
14. Valenta R. The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002;2:446-53.
15. Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol* 2003;130:87-107.
16. Mari A. Skin test with a timothy grass (*Phleum pratense*) pollen extract vs. IgE to a timothy extract vs. IgE to rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, and rPhl p 12: epidemiological and diagnostic data. *Clin Exp Allergy* 2003;33:43-51.
17. Bergmann RL, Bergmann KE, Lau-Schadensdorf S, Luck W, Dannemann A, Bauer CP, et al. Atopic diseases in infancy. The German multicenter atopy study (MAS-90). *Pediatr Allergy Immunol* 1994;5(suppl):19-25.
18. Asher MI, Keil U, Anderson HR, Beasley R, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J* 1995;8:483-91.
19. Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM, Twardosz A, et al. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J* 2002;16:414-6.
20. Petersen A, Bufe A, Schlaak M, Becker VM. Characterisation of the allergen group VI in timothy grass (Phl p 6). I. Immunological and biochemical studies. *Int Arch Allergy Immunol* 1995;108:49-54.
21. Kellberger J, Dressel H, Vogelberg C, et al. Prediction of the incidence and persistence of allergic rhinitis in adolescence: a prospective cohort study. *J Allergy Clin Immunol* 2012;129:397-402, e1-3.
22. Schäfer T, Wölke G, Ring J, Wichmann HE, Heinrich J. Allergic sensitization to cat in childhood as major predictor of incident respiratory allergy in young adults. *Allergy* 2007;62:1282-7.
23. Rossi RE, Monasterolo G, Monasterolo S. Measurement of IgE antibodies against purified grass-pollen allergens (Phl p 1, 2, 3, 4, 5, 6, 7, 11, and 12) in sera of patients allergic to grass pollen. *Allergy* 2001;56:1180-5.
24. Dolecek C, Vrtala S, Laffer S, Steinberger P, Kraft D, Scheiner O, et al. Molecular characterization of Phl p II, a major timothy grass (*Phleum pratense*) pollen allergen. *FEBS Lett* 1993;335:299-304.
25. Eifan AO, Shamji MH, Durham SR. Long-term clinical and immunological effects of allergen immunotherapy. *Curr Opin Allergy Clin Immunol* 2011;11:586-93.
26. Valenta R, Twaroch T, Swoboda I. Component-resolved diagnosis to optimize allergen-specific immunotherapy in the Mediterranean area. *J Investig Allergol Clin Immunol* 2007;17(suppl):36-40.
27. Melioli G, Marcomini L, Agazzi A, Bazurro G, Tosca M, Rossi GA, et al. The IgE repertoire in children and adolescents resolved at component level: a cross-sectional study. *Pediatr Allergy Immunol* 2012;23:433-40.
28. Chabre H, Gouyon B, Huet A, Baron-Bodo V, Nony E, Hrabina M, et al. Molecular variability of group 1 and 5 grass pollen allergens between Pooideae species: implications for immunotherapy. *Clin Exp Allergy* 2010;40:505-19.
29. Rudenko M, Frew AJ. How important is it to include all epitopes in grass pollen extracts for specific immunotherapy? *Clin Exp Allergy* 2010;40:365-7.
30. Johansen N, Weber RW, Ipsen H, Barber D, Broge L, Hejl C. Extensive IgE cross-reactivity towards the Pooideae grasses substantiated for a large number of grass-pollen-sensitized subjects. *Int Arch Allergy Immunol* 2009;150:325-34.
31. Westritschnig K, Horak F, Swoboda I, Balic N, Spitzauer S, Kundi M, et al. Different allergenic activity of grass pollen allergens revealed by skin testing. *Eur J Clin Invest* 2008;38:260-7.
32. Weber RW. Patterns of pollen cross-allergenicity. *J Allergy Clin Immunol* 2003;112:229-39.

METHODS

In short, 499 newborns with risk factors for atopy (ie, increased cord blood IgE level [≥ 0.9 kU_A/L], at least 2 atopic family members, or both) and 815 newborns with none of these risk factors were included in the cohort. All children were followed up at ages 1, 3, 6, 12, 18, and 24 months and from then on an annual basis within 4 weeks of the child's birthday up to the age of 13 years. At each follow-up, parents gave structured interviews to a study physician, which included standardized questions on asthma and atopy symptoms according to the International Study of Asthma and Allergy in Childhood.^{E1}

SARg onset was calculated as the first observed time point with SARg symptoms. In a few cases only ($n = 17/177$ [9.6%]), this first time point was preceded by a missing value that has been arbitrarily considered a negative value.

Information on SIT with grass pollen, birch pollen, or both extracts has been obtained from 707 (86.2%) of the 820 children. Overall, 17 (2.4%) of 707 children received specific immunotherapy at or before 13 years of age against grass pollen only ($n = 12$) or combined with other pollens ($n = 5$). Most subjects ($n = 13$) started specific immunotherapy after 9 years of age.

ISAC (Thermo Fisher Scientific) is based on 103 recombinant and natural purified allergens spotted in triplicate on a glass slide. The testing procedures have been carried out according to the manufacturer's instructions. During assay performance, at first, slides were washed for 60 minutes in washing solution, dried, and placed into a humid chamber. Subsequently, 20 μ L of serum from IgE-positive children (IgE ≥ 0.35 kU_A/L) was applied to each chip of the slide. Incubation at room temperature was followed by a second washing procedure in washing solution and water. For the detection of IgE serum antibodies bound on the spotted allergens of the chip, 20 μ L of a fluorescence-labeled anti-human IgE antibody was applied on the chip. After incubation, chips were again washed, dried, and stored protected from light. Data acquisition was performed with an appropriate microarray scanner (Lux-Scan-10K/A; CapitalBio, Beijing, China). Allergens arrayed in triplicates become visible in false color display mode. Analysis of IgE concentration was performed by using the Microarray Image Analyser software, evaluating the fluorescence of labeled anti-IgE antibodies by means of a calibration curve created based on a standardized reference serum. Thereby the IgE concentrations were expressed semiquantitatively as ISAC Standard Units (ISUs) in 4 classes: undetectable or very low (<0.3 ISU), low (≥ 0.3 - <1 ISU), moderate to high (≥ 1 - <15 ISU), and very high (≥ 15 ISU). A result of greater than 0.3 ISU was considered positive.

RESULTS

The frequency distribution of missing values, as stratified by follow-up points, is shown in Table E1. Information on the natural history of allergic rhinitis was obtained in more than 90% of the included children up to the follow-up at age 13 years. On average, 539 of 820 blood samples were obtained at each follow-up visit, with a minimum of 421 (51.3%) of 820 at age 13 years and a maximum of 610 (74.4%) of 820 at age 7 years. In all, 611 (84.0%) of 727 of the eligible sera (because they were positive for IgE against *P pratense* extract) were still available and could be tested with the microarray IgE test for molecules. IgE sensitization could already be observed 1 or more years before the onset of a reported

disease in 77 (45.8%) of the 168 patients for whom this analysis was possible; this proportion would have probably been higher if all the subjects had provided blood samples at all the follow-up points.

Phl p 1 is the only recognized allergen at the first detection of molecular sensitization in 26.2% of the 126 patients with SARg tested with ISAC. Phl p 1 was undetectable in only 31 (24.8%) of the 126 cases in the first positive and examined sample. Conversely, Phl p 12 was never detected, and Phl p 7 and Phl p 11 were detected only once in the first positive and tested sample. Phl p 4 has been detected often (together with Phl p 1 or alone) as a first recognized allergen. However, this native allergen in the ISAC test is the only one with carbohydrate moieties and might have produced false-positive outcomes.

DISCUSSION

It can be argued that in Germany there are other pollens that are prevalent in June and July and could be responsible for symptoms of allergic rhinitis (eg, rye grass [*Lol p 1-3*]). *Lolium perenne* and other grass pollen species contain allergens largely cross-reacting with *P pratense*, which was chosen in this study as an index species. The trends observed in this study are therefore valid for grass pollen in general, although obtained by studying only 1 representative member (ie, *P pratense*).

On the other hand, we cannot exclude an influence on the molecular sensitization profile because of other pollens (eg, birch and mugwort) containing polcalcins and profilins. However, the prevalence of sensitization to these pollens is relatively low. Profilins (Phl p 12) and polcalcins (Phl p 7) were the least and latest molecules recognized based on IgE measurements in the examined children. Therefore even by accounting for the potential influence of other pollens, the position in the sequence of sensitization of these 2 molecules would not have substantially changed.

To define SARg, we adopted an accepted epidemiologic diagnosis based on answers to 2 questions only: (1) nasal symptoms apart from colds and (2) occurrence of these symptoms in June, July, or both. This epidemiologic definition is less specific than a clinical one, and false positivity because of nonspecific responses is possible. In addition, the parents obviously received information on the allergy test results. This might have influenced the reporting of SARg. It is well known that participation in a cohort study influences disease perception.

REFERENCE

- E1. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J* 1995;8:483-91.

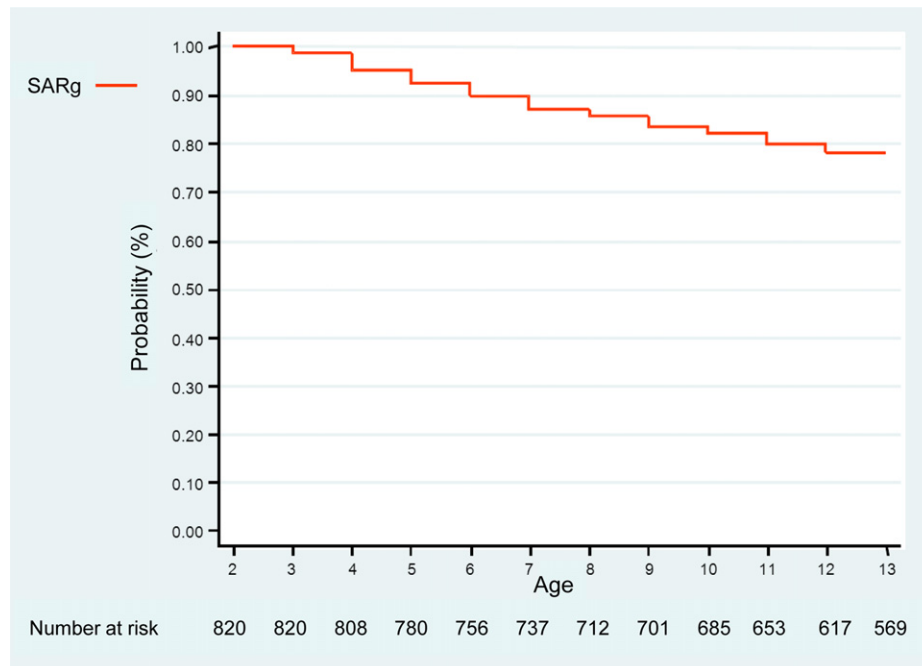


FIG E1. Probability of remaining free of seasonal allergic rhinitis related to grass pollen. Kaplan-Meier plots of the probability of remaining free of seasonal allergic rhinitis related to grass pollen among 820 children participating in the MAS birth cohort are shown. The number of subjects at risk is shown below the *x-axis*.

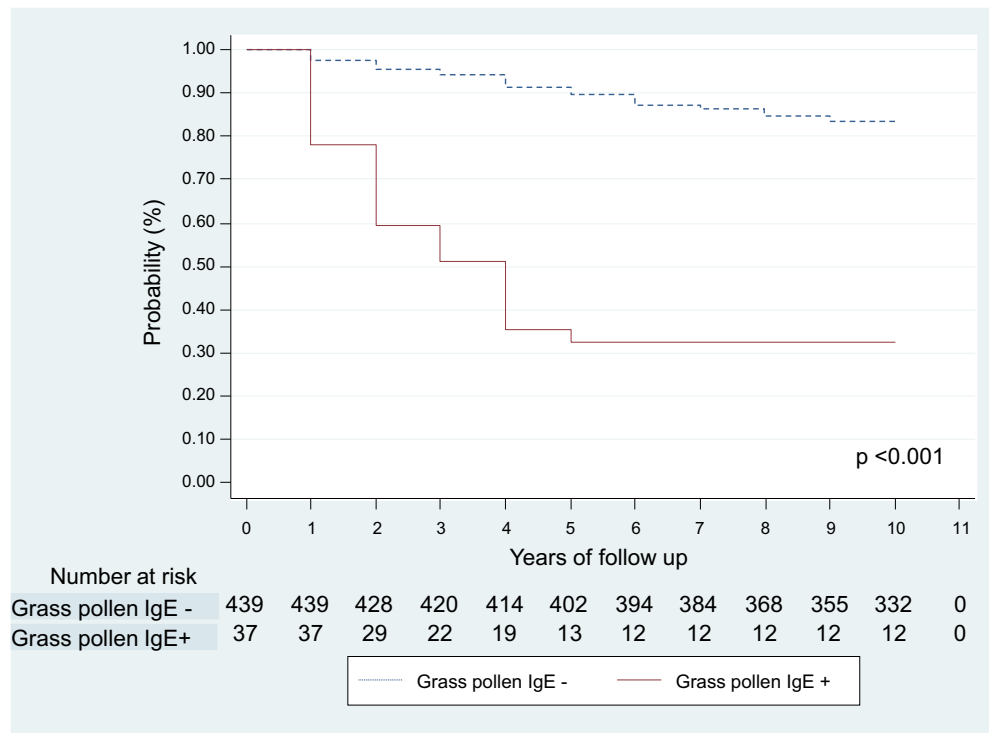


FIG E2. Probability of remaining free of seasonal allergic rhinitis related to grass pollen at age 3 years by IgE sensitization to *P pratense* (extract). Kaplan-Meier plots of the probability to remain free of seasonal allergic rhinitis related to grass pollen stratified by IgE sensitization to grass pollen at age 3 years are shown.

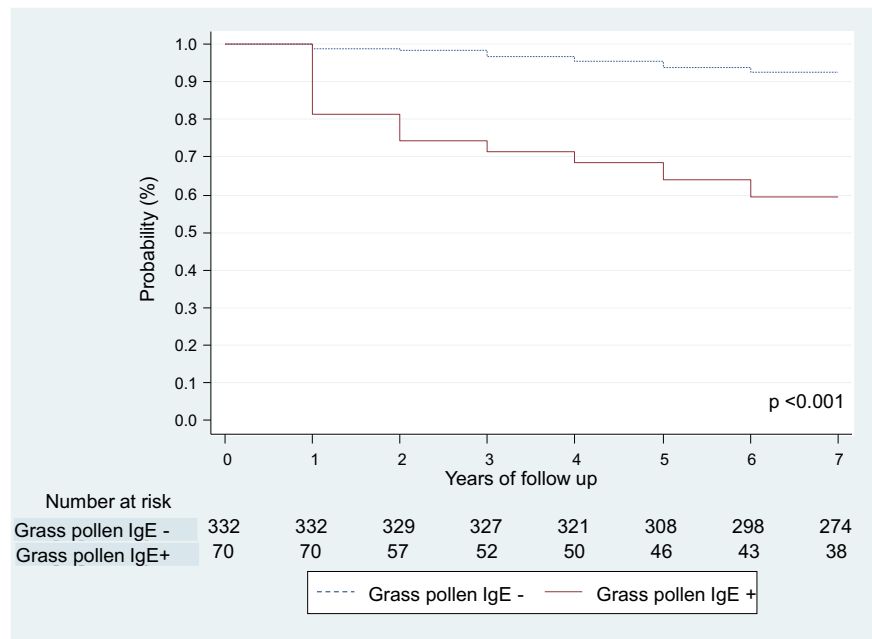


FIG E3. Probability of remaining free of seasonal allergic rhinitis related to grass pollen by IgE sensitization to *P pratense* (extract). Kaplan-Meier plots of the probability at 6 years of age of remaining free of seasonal allergic rhinitis related to grass pollen stratified by IgE sensitization to grass pollen are shown. The number of subjects examined at each follow-up point is reported under the x-axis.

TABLE E1. Availability of questionnaire data, blood samples, and IgE test results

Variable	Age (y)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Participating	820	820	820	820	820	820	820	820	820	820	820	820	820
Questionnaire data	820	820	820	818	816	814	815	804	803	798	758	763	731
Blood samples and IgE tests (extract)	581	517	482	NA	548	453	610	NA	NA	561	NA	NA	421
Eligible for microarray*	7	37	38	NA	97	102	145	NA	NA	162	NA	NA	139
Available for molecular IgE tests†	7	18	29	NA	84	89	129	NA	NA	155	NA	NA	100

NA, Not applicable (no blood drawn at this follow-up).

*IgE level of 0.35 kU_A/L or greater against *P pratense* (extract).

†Sera still available at the time of microarray testing.