

ORIGINAL ARTICLE

WILEY

Airways Disease

Sublingual immunotherapy alters salivary IgA and systemic immune mediators in timothy allergic children

Johanna Huoman¹  | Georgia Papapavlou¹ | Anna Pap¹ | Johan Alm² |
Lennart J. Nilsson³  | Maria C. Jenmalm¹ 

¹Division of Neuro and Inflammation Sciences, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

²Karolinska Institutet, Department of Clinical Science and Education, Sachs' Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden

³Allergy Center, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

Correspondence

Johanna Huoman, Department of Clinical and Experimental Medicine, Division of Neuro and Inflammation Sciences (NIV), Unit for Autoimmunity and Immune regulation (AIR), Faculty of Medicine and Health Sciences, Linköping, Sweden.
Email: johanna.huoman@liu.se

Funding information

The study was supported by grants from the Research Council for South-East Sweden and the University Hospital of Linköping, Sweden.

Edited by: Ömer Kalaycı

Abstract

Background: Immunomodulatory effects of sublingual immunotherapy on systemic and mucosal mediators in allergic children are largely unexplored. The aim of this study was to investigate allergy-related cytokine and chemokine levels, as well as IgA-responses upon a 3-year treatment with timothy grass pollen sublingual immunotherapy in children with allergic rhinoconjunctivitis.

Methods: From children included in the GRAZAX[®] Asthma Prevention study, blood and saliva samples were analyzed at inclusion, after 3 years of treatment, and 2 years after treatment ending. By means of Luminex and ELISA methodologies, allergy-related cytokines and chemokines were measured in plasma samples and allergen-stimulated peripheral blood mononuclear cell supernatants. Furthermore, studies of total, secretory, and Phl p 1-specific salivary IgA antibodies were performed using the same methods.

Results: GRAZAX[®]-treated children exhibited significantly higher levels of Phl p 1-specific salivary IgA and serum IgG₄, along with significantly lower skin prick test positivity, after 3 years of treatment and 2 years after treatment cessation. Additionally, plasma levels of the Th1-associated chemokines CXCL10 and CXCL11 were significantly higher in treated than untreated children at these time points. Timothy-induced ratios of IL-5/IL-13 over IFN- γ were significantly decreased after 3 years with active treatment, as were symptoms of allergic rhinitis in terms of both severity and visual analogue scale scores. However, no consistent correlations were found between the clinical outcomes and immunologic parameters.

Conclusion: *Phleum pratense* sublingual immunotherapy in grass pollen allergic children modulates the immune response in the oral mucosa as well as systemically—by increasing Th1-responses, decreasing Th2-responses, and inducing immunoregulatory responses—all signs of tolerance induction.

KEYWORDS

allergy, chemokines, children, cytokines, IgA, immunomodulation, immunotherapy, *Phleum pratense*, saliva, sublingual, timothy grass

1 | INTRODUCTION

The prevalence of allergic diseases including allergic rhinoconjunctivitis (ARC) and allergic asthma has in recent decades increased among children and adolescents,¹ affecting quality of life, sleeping patterns, and school performance, along with high socioeconomic costs.² Furthermore, it is well established that prevalence of ARC predisposes for development of allergic asthma,^{3,4} pointing at the importance of intervening at an early time point. As today's treatment options for ARC mainly comprise symptomatic pharmacotherapy often accompanied by side effects, there is a great need of better treatment options. Allergen-specific immunotherapy (AIT) is the only treatment approach that may cure individuals from allergic disease, by reconstructing tolerance toward the allergen they are sensitized against.^{5,6} Throughout treatment, proportional changes of Th2 cells over Th1- and Treg-responses may shift the immune imbalance toward a more tolerant state. Immunologic effects of AIT involve remission of present allergen-specific immune responses, by means of anergy, apoptosis, and inhibition of T cells, along with induction of immunoregulatory responses in both the innate and adaptive immune system. The number and activity of innate immune cells such as basophils, mast cells, and eosinophils decrease, and induction of Tregs and their production of IL-10 and TGF- β modulate humoral responses toward production of blocking antibodies such as IgA and IgG₄. Altogether, this shifts the immune response away from IgE-dependent mast cell activation and related allergic symptoms.^{5,6}

Subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) are clinically available administration routes of AIT, and both prove efficacious for the treatment of seasonal allergic rhinitis.⁷ However, due to the tedious process of SCIT treatment, SLIT is considered a patient-convenient option, because of self-administration at home after administration of an initial dose under clinical surveillance. Additionally, SLIT is superior to SCIT regarding tolerability and safety, as a result of lower treatment doses as well as fewer and less severe side effects.⁷ Generally, more is known about immunomodulatory effects of SLIT in adults, displaying induction of immunomodulatory effects on both innate and adaptive immune responses,^{8,9} whereas children and adolescents still remain poorly investigated. Recently, AIT has also been examined as an allergy preventive option in children.¹⁰⁻¹² For instance, children with birch- and/or grass-related ARC treated with SCIT for three consecutive years¹¹ had less ARC symptoms and lower risk of developing allergic asthma compared to untreated children throughout and after treatment ending in the open follow-up of the study.¹⁰⁻¹² More recently, the GRAZAX[®] Asthma Prevention (GAP) study investigated possible asthma-preventive effects of timothy grass (*Phleum pratense*) SLIT in children with timothy grass-related ARC.¹³ Less ARC and asthma symptoms, and lower medication scores, were recorded in the actively vs. placebo-treated individuals throughout treatment and two years after treatment cessation.⁴ However, the primary outcome of differences in time to onset of asthma was not met, which may be due to that reversibility of lung function is not as reliable a marker in children as in adults for asthma development.⁴ Moreover, total and specific IgE levels

Key Message

Mucosal and systemic immunological effects of sublingual immunotherapy in grass pollen allergic children are largely unexplored. In a three-year timothy grass pollen sublingual immunotherapy placebo-controlled intervention study, actively treated subjects revealed a shift towards a balance between Th2 and Th1 responses. By inducing e.g. grass pollen-specific IgA in saliva and elevated circulating levels of Th1-associated chemokines in actively treated children, a more tolerant state was observed at both the mucosal and systemic level, with effects persisting two years after treatment end. These findings could provide novel biomarkers for studying treatment success.

as well as skin prick test (SPT) positivity were lower and specific IgG₄ levels were higher in actively as compared with placebo-treated subjects, indicating long-term immunomodulating effects of the treatment on the immune system. However, further immune effects of SLIT in grass pollen allergic children need to be characterized.

As SLIT is administrated orally, the first encounter with the allergen takes place in the oral cavity mucosa, leading to local induction of allergen-specific IgA production by resident B cells but also immunomodulation at other immune reactive sites.⁷ In the mucosa, IgA mainly exists as homodimers protected from enzymatic degradation by the secretory component. Primarily, it performs its functions by means of immune exclusion, by binding antigens and preventing induction of immune responses at mucosal sites.^{14,15} Previously, we have shown that sensitized children with high levels of secretory IgA in saliva at 1 year of age were less likely to develop late-onset wheezing at the age of 4.¹⁶ Moreover, sensitized infants with allergic symptoms throughout the first 2 years of life had lower levels of secretory IgA in the first year of life than sensitized children without symptoms,¹⁷ altogether suggesting a protective effect of IgA to development of allergic symptoms. Children with timothy grass-induced ARC are most commonly sensitized toward the component Phl p 1,^{18,19} but studies on Phl p 1-specific IgA are scarce. AIT may additionally affect systemic mediators such as cytokines^{5,6} and chemokines. Previous studies by us revealed that cord blood levels of the Th2-associated chemokines CCL17 and CCL22 were related to allergic symptoms and sensitization, respectively, during childhood.^{20,21} Moreover, increased CCL17 and CCL22 vs. CXCL10 ratios were observed in children developing allergies, suggesting a Th2-skewing in allergic children.²² How SLIT affects levels of allergy-related cytokines and chemokines in the circulation as well as *in vitro* has not been well studied. Furthermore, the effects of SLIT on salivary levels of total, secretory, and Phl p 1-specific IgA remain poorly understood, highlighting the importance of the present study.

In this sub-study of the GAP study, we aimed at investigating the role of timothy grass pollen SLIT (GRAZAX[®]) in immunomodulation of manifested grass pollen-induced ARC in children treated with GRAZAX[®]

TABLE 1 Descriptive data of the children from the study sites in Linköping and Stockholm, Sweden, recruited in the GAP study

		PLACEBO % (n/N)			GRAZAX % (n/N)	
Gender (girls)		40 (6/15)			30 (3/10)	
Age at inclusion, mean (youngest to oldest)		9.2 (7-12)			9.6 (7-12)	
History of eczema		40 (6/15)			50 (5/10)	
History of food allergy		7 (1/15)			10 (1/10)	
	Inclusion	3 y	5 y	Inclusion	3 y	5 y
Other sensitizations, % SPT positive (n/N)						
Birch— <i>Betula verrucosa</i>	60 (9/15)	100 (10/10)	80 (8/10)	50 (5/10)	57 (4/7)	38 (3/8)
Weed— <i>Artemisia vulgaris</i>	13 (2/15)	30 (3/10)	30 (3/10)	10 (1/10)	0 (0/7)	0 (0/8)
Horse— <i>Equus ferus</i>	0 (0/15)	0 (0/10)	20 (2/10)	30 (3/10)	29 (2/7)	38 (3/8)
Dog— <i>Canis familiaris</i>	20 (3/15)	40 (4/10)	10 (1/10)	0 (0/10)	29 (2/7)	13 (1/8)
Cat— <i>Felis domesticus</i>	33 (5/15)	40 (4/10)	40 (4/10)	60 (6/10)	57 (4/7)	50 (4/8)
House Dust Mites— <i>Dermatophagoides pteronyssinus</i>	0 (0/15)	10 (1/10)	10 (1/10)	0 (0/10)	14 (1/7)	0 (0/8)
House Dust Mites— <i>Dermatophagoides farinae</i>	0 (0/15)	0 (0/9)	0 (0/10)	0 (0/10)	13 (1/8)	0 (0/8)
Molds— <i>Alternaria alternata</i>	0 (0/15)	0 (0/10)	0 (0/10)	10 (1/10)	0 (0/7)	13 (1/8)
Molds— <i>Cladosporium herbarium</i>	0 (0/15)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/7)	0 (0/8)
Other concomitant allergic symptoms at time of inclusion, % (n/N)						
Atopic dermatitis	40 (6/15)			50 (5/10)		
Urticaria	7 (1/15)			0 (0/10)		
Eczema	7 (1/15)			10 (1/10)		
Birch pollen allergy	7 (1/15)			10 (1/10)		
Food allergy	7 (1/15)			10 (1/10)		
Allergy to furred pets	7 (1/15)			30 (3/10)		
	Family history of allergic disease					
	Father	Mother	Sibling	Father	Mother	Sibling
Asthma	7 (1/15)	13 (2/15)	33 (5/15)	20 (2/10)	10 (1/10)	22 (2/9) ^a
ARC	80 (12/15)	53 (8/15)	67 (10/15)	50 (5/10)	60 (6/10)	44 (4/9) ^a

Differences between the groups in categorical variables were investigated by means of Fisher's exact test, due to the low observation counts in the groups. Differences in continuous variables were examined by means of Student's *t* test. No differences were detected for any of the variables between the intervention groups. In the placebo group, five individuals were lost to follow-up from inclusion to the later time points. In the GRAZAX[®] group, three individuals were lost to follow-up at the 3-y time point, whereas only two individuals were lost to follow-up 2 y after the treatment was ceased.

^aInformation missing from one child without siblings.

for three consecutive years. We hypothesized that treatment with GRAZAX[®] will modulate the immune response of treated subjects toward Th1- and Treg-induction along with decreased Th2-responses. Additionally, we hypothesized that the levels of Phl p 1-specific IgA in saliva will increase in treated as compared to non-treated children.

2 | METHODS

The present study utilized samples from the international multi-center GRAZAX[®] Asthma Prevention (GAP) study, a randomized, double-blind, placebo-controlled intervention study aiming at investigating possible asthma-preventive effects of grass pollen-specific

immunotherapy in children with current grass pollen-induced ARC.¹³ For additional information on the study participants, we refer to previous publications^{4,13} and Appendix S1. From the total of 812 individuals included in the entire study,⁴ 28 children were included from two study sites in Sweden (Linköping and Stockholm). Of these children, 17 completed the study and blood and saliva samples were available from 25 unique individuals. The active treatment consisted of lyophilized SQ sublingual tablets containing standardized timothy grass pollen allergen extracts from *Phleum pratense* (GRAZAX[®], ALK, Hørsholm, Denmark), whereas the placebo treatment consisted of a sublingual tablet without grass pollen extract, in appearance non-distinguishable from the active treatment. Upon inclusion in the study, the participants were administering the tablet sublingually once daily for

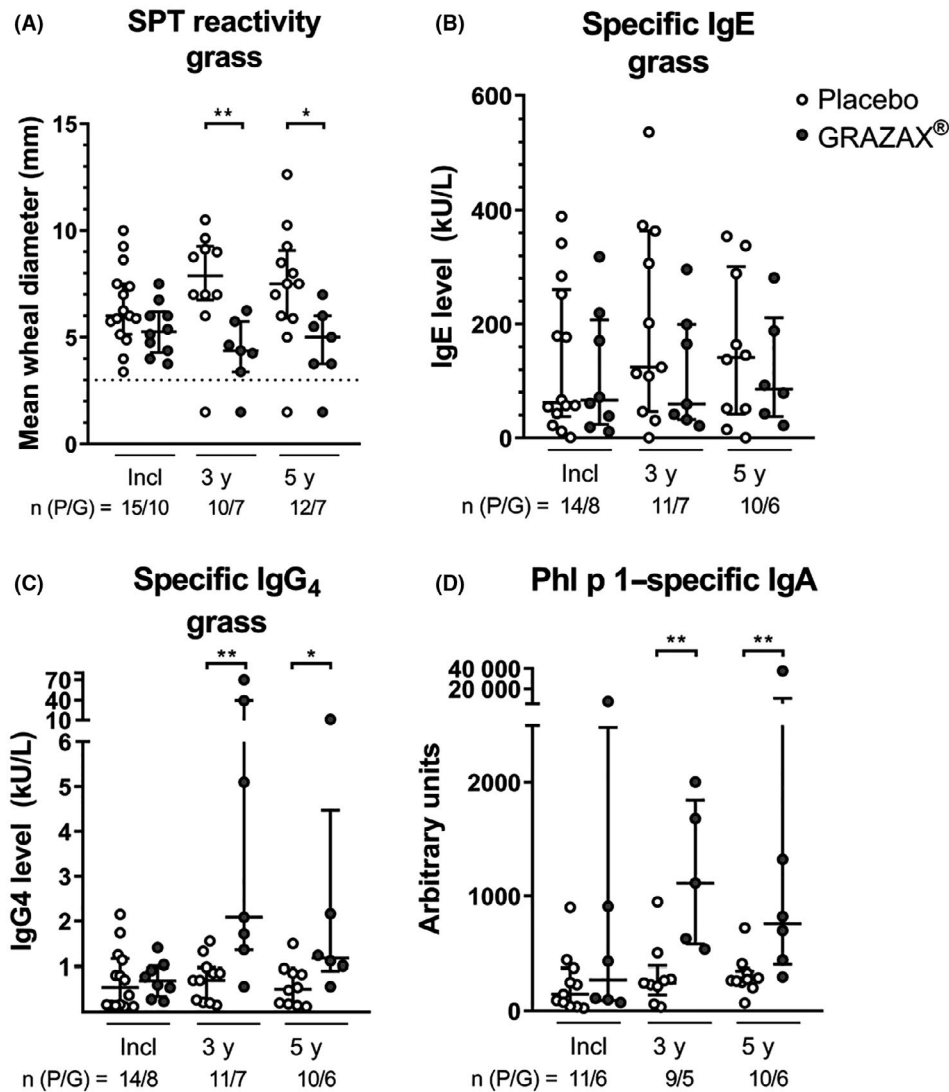


FIGURE 1 Skin prick test (SPT) reactivity and levels of timothy grass pollen-specific antibodies in serum and saliva. Mean wheal diameter (in mm) upon skin prick testing with grass pollen allergen (A) and grass pollen-specific IgE levels in serum (B) were utilized as measures of sensitization toward the timothy grass allergen *Phleum pratense*. The dotted line in (A) indicates the cutoff for SPT positivity. The induction of possible IgE-blocking effects was measured by investigating the levels of grass pollen-specific IgG₄ in serum (C). Mucosal effects of GRAZAX® were investigated by analyzing the levels of Phl p 1 (timothy grass pollen component)-specific IgA in saliva, as depicted in (D). All measurements were performed at inclusion, after 3 y of treatment, and 2 y after treatment ending (5 y from inclusion time point). White circles indicate placebo treatment and gray circles GRAZAX® treatment. The numbers of individuals analyzed are indicated below each time point as n (P = Placebo/G = GRAZAX®). The data are illustrated as medians with interquartile ranges. * $P < 0.05$, ** $P < 0.01$, Mann-Whitney U test

three consecutive years, followed by a 2-year open follow-up period. Informed consent was obtained from parents or legal guardians of the children. The study was approved by the Regional Ethics Committee for Human Research in Linköping, Sweden (Dnr 2010/430, 2009/551, 2009-0111235-12, 151:2010/48756).

3 | SAMPLE COLLECTION, EXPERIMENTAL PROCEDURES, AND STATISTICS

Blood and saliva samples were prepared and analyzed at inclusion (prior to treatment, n Blood/Saliva = 20/17), after 3 years with treatment (n

Blood/Saliva = 15/14), and 2 years after treatment ended (n Blood/Saliva = 15/16). For details on experimental procedures and statistical analyses, see Appendix S1. Briefly, PBMCs were isolated from peripheral blood and stimulated with grass and birch pollen allergens. Supernatants from these experiments were analyzed by means of ELISA (IL-10) and Luminex (IFN- γ , IL-5, IL-10, IL-13, CCL17, and CXCL10) methodologies, to examine content of allergy-related cytokines and chemokines. Additionally, plasma samples were retrieved from the blood samples and analyzed regarding cytokine and chemokine content with ELISA (CCL18) and Luminex (CCL17, CCL22, CXCL10, and CXCL11). Saliva samples were isolated and heat-inactivated prior to analysis of total and secretory IgA (ELISA) as well as Phl p 1-specific

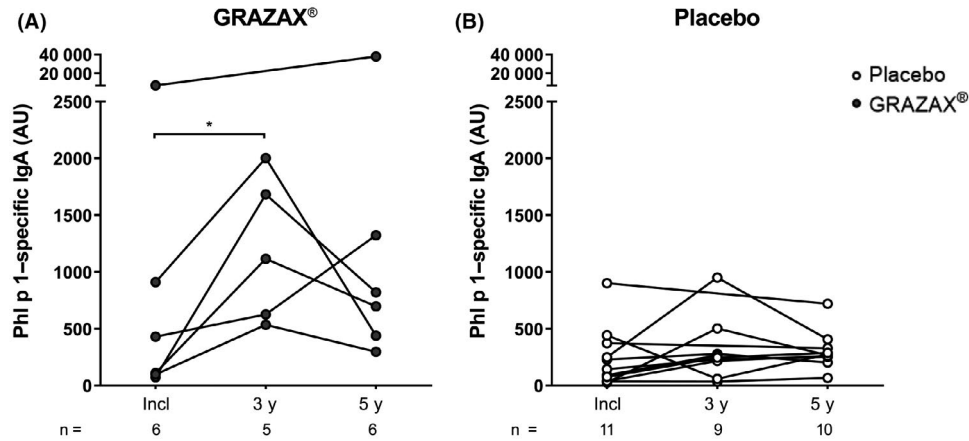


FIGURE 2 The levels of Phl p 1 (timothy grass pollen component)-specific IgA in saliva as studied over time. A Friedman test with Dunn's multiple comparisons test was performed separately for (A) the GRAZAX® group (n = 5) and (B), the placebo group (n = 8) for individuals having samples available for all time points. All measurements were performed at inclusion, after 3 y of treatment, and 2 y after treatment ending (5 y from inclusion time point). Gray circles indicate GRAZAX® treatment, white circles depict placebo treatment. The total number of individuals in the groups at the respective time points is indicated below each time point. **P* < 0.05

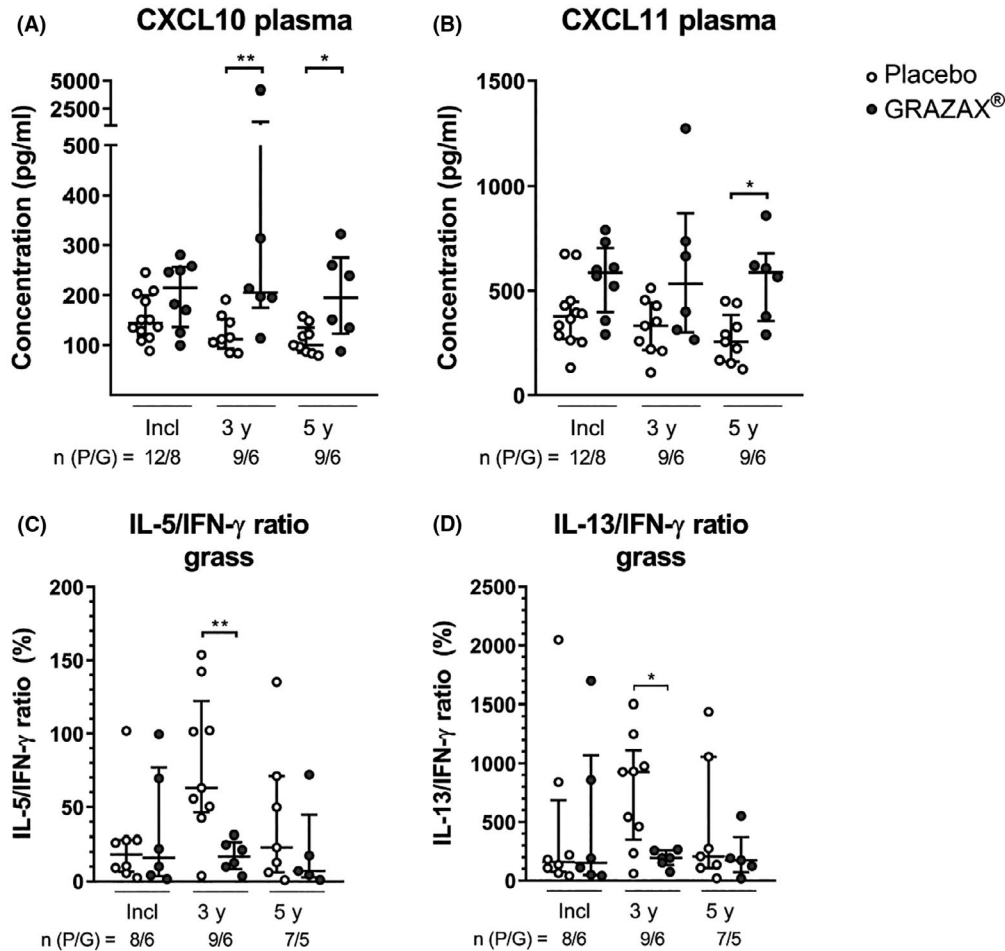


FIGURE 3 Plasma and supernatant levels of T helper cell-associated chemokines and cytokines. Circulating levels of the Th1-associated chemokines CXCL10 (A) and CXCL11 (B) were measured in plasma, whereas the levels of IL-5, IL-13, and IFN-γ were analyzed in supernatants from PBMCs stimulated with grass allergen for 6 days. The ratio of Th2- over Th1-associated cytokines may be used as a surrogate mirroring the balance of the two immune responses in the immune system, as shown for IL-5/IFN-γ in (C), and IL-13/IFN-γ in (D). All measurements were performed at inclusion, after 3 y of treatment, and 2 y after treatment ending (5 y from inclusion time point). White circles indicate placebo treatment and gray circles GRAZAX® treatment. The numbers of individuals analyzed are indicated below each time point as n (P = Placebo/G = GRAZAX®). The data are illustrated as medians with interquartile ranges. **P* < 0.05, ***P* < 0.01, Mann-Whitney *U* test

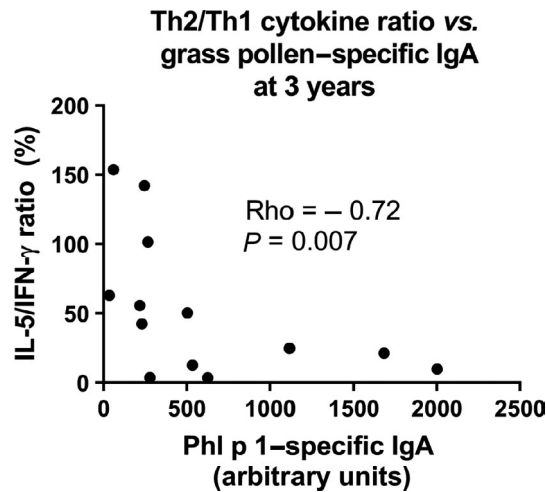


FIGURE 4 Phl p 1-specific IgA levels in saliva in correlation with the T helper cell-associated cytokine IL-5/IFN- γ ratio in grass pollen allergen-stimulated PBMC supernatants originating from the time point after 3 y of treatment. Spearman's rank correlation test was utilized for the analysis

IgA (1-plex Luminex) levels. Data from skin prick tests (SPTs), IgG₄-, and IgE-levels were collected at clinical follow-up visits throughout the treatment period.

4 | RESULTS

In order to investigate whether immunomodulation had taken place in GRAZAX[®]-treated individuals, analyses on several levels of the immune system were carried out. Demographic comparisons between the intervention groups did not reveal any statistical differences (Table 1).

Examination of SPT reactivity for timothy grass demonstrated significantly lower mean wheal diameters in children from the active compared to the placebo group, both immediately after treatment cessation and 2 y later (Figure 1A). However, this pattern was not reflected in circulating levels of grass pollen-specific IgE (Figure 1B). Additionally, SPT reactivity toward birch was tentatively lower in children treated with SLIT at the 3-year time point (Figure S1). Elevated levels of *P. pratense*-specific IgG₄ antibodies were observed at the two later time points among GRAZAX[®]-treated children (Figure 1C).

Since the intervention was administered sublingually, effects of GRAZAX[®] on the local mucosal immune system were studied. Salivary levels of IgA antibodies toward the timothy grass pollen component Phl p 1 were significantly higher in the active than the placebo group after 3 y of treatment and 2 y after treatment ending (Figure 1D). The levels of Phl p 1-specific IgA in saliva also increased significantly from inclusion to the 3-year time point within the active (Figure 2A), but not within the placebo group (Figure 2B). Total and secretory IgA levels in saliva were similar in both groups (Figure S2).

Systemic effects of SLIT were investigated in both plasma and supernatants of allergen-stimulated PBMCs. In plasma samples,

levels of the Th1-associated chemokines CXCL10 and CXCL11 were consistently higher in relation to the placebo group, significantly so after 3 years with active treatment for CXCL10 (Figure 3A) and maintained at these levels 2 years after treatment ended for both CXCL10 and CXCL11 (Figure 3B). No differences were observed for the chemokines CCL17, CCL22, and CCL18 between the groups, neither were there any differences in the levels of the cytokines and chemokines (IFN- γ , IL-5, IL-10, IL-13, CCL17, and CXCL10) measured in supernatants of grass or birch pollen-stimulated PBMCs (Table S1). However, when studying the Th2/Th1-ratio of the cytokines IL-5 and IL-13 over IFN- γ levels in grass pollen-stimulated cell supernatants, significantly lower IL-5/IFN- γ and IL-13/IFN- γ ratios were observed in the active than the placebo group at the 3-year time point (Figure 3C,D). Moreover, at the 3-year time point, the grass-stimulated supernatant IL-5/IFN- γ ratio was strongly inversely correlated with the levels of Phl p 1-specific IgA antibodies in saliva (Figure 4, Rho = -0.72, P = 0.007).

Investigations of the relationship between immunologic markers and symptoms of allergic rhinitis revealed that in the grass pollen season (GPS) in conjunction to the 3-year time point, significantly lower visual analogue scale (VAS) scores (Figure S3A) as well as allergic rhinitis severity (ARS) scores (Figure S3B) were recorded in the GRAZAX[®] compared to the placebo group. Furthermore, in peak to end of the GPS closest to the 5-year follow-up, the mean values of 14-day VAS recordings were significantly lower in the treated compared to the untreated group (Figure S3C). Correlation analyses of clinical outcomes, in terms of VAS and ARS scores, and immunologic markers revealed no consistent patterns (results not shown).

5 | DISCUSSION

The main findings of this paper were that timothy grass SLIT induces specific immune-modulating effects on the local immune environment in the oral mucosa and on circulating Th1-associated chemokines in treated subjects. Being the main allergen component toward which children are sensitized,^{18,19} IgA antibodies in saliva specific for Phl p 1 were significantly higher in individuals treated with GRAZAX[®] for 3 years compared to the control group. This is in line with findings from a mixed age group SCIT cohort of house dust mite allergic individuals, in which salivary levels of Der p 1-specific IgA were increased in actively treated subjects.²³ Additionally, in the present study we observed a sustained effect 2 years after treatment cessation, suggesting that B cells have become IgA producers in the local immune environment and possibly remained as memory cells after the treatment ended. This is in line with findings from adults treated with grass pollen SLIT (droplets), showing the presence of more FoxP3+ cells in the oral mucosa in peak season and after 12-18 months, indicating induction of long-term immunoregulatory responses.⁸ Despite the decrease in the levels of Phl p 1-specific IgA after 5 years, it also appears that these levels are still sufficient to maintain tolerance in both the local and general immune environment, as indicated by the maintained treatment effect after cessation.⁴ Total and secretory IgA levels in saliva did not

differ between the groups, which could be explained by the total IgA making up a mixed proportion of specificities toward a plethora of antigens and allergens. In line with results from the GAP trial,⁴ GRAZAX[®]-treated subjects rated their ARC symptoms significantly lower than untreated subjects. This is evident by the lower VAS scores in the GPS closest to the end of treatment as well as by the lower mean VAS scores of recorded 14-day data prior to the final follow-up, indicating long-term effects also on perceived symptom severity. The lack of clear correlation patterns between allergic rhinitis symptoms and the assayed immunologic variables most probably originates from the limited power of these analyses.

Similar to the levels of Phl p 1-specific IgA in saliva, the circulating levels of specific IgG₄ were significantly elevated in the active compared to the placebo group after 3 years with treatment and at the 2-year follow-up, in line with previous findings from the main study.⁴ IgG₄ may in the context of AIT be considered immunomodulatory, as it upon Fab arm exchange does not form immune complexes, generally does not activate complement, and it competes with other antibody isotypes, such as IgE, for allergen binding.²⁴ Furthermore, IgG₄ antibodies are the least abundant IgG subclass, with distinct increases upon AIT,²⁴ indicating that the levels of allergen-specific IgG₄ antibodies increase upon induction of tolerance. Conflicting results from adults stand in contrast to our findings in children; in adults treated with GRAZAX[®] tablets, levels were elevated during the treatment period but not thereafter.^{9,25} However, in line with our results, a study with *P. pratense* droplets showed elevation of specific IgG₄ both during and after treatment.⁸ This was further corroborated by findings in serum samples from actively treated subjects, which significantly inhibited IgE-mediated allergen binding, indicating the presence of blocking antibodies.⁸ SPT reactivity toward timothy grass was lower after 3 years of treatment as well as 2 years after treatment ending in GRAZAX[®]-treated compared to untreated children. The latter finding is in line with results from the main study,⁴ which indicates a reduction in the activity of innate immune cells such as mast cells and basophils in individuals treated with GRAZAX[®]. However, in contrast to the main study,⁴ we could not observe a mirroring of this effect in the levels of circulating allergen-specific IgE, possibly due to the small sample size. We also found a trend in the reduction of SPT reactivity toward birch, possibly suggesting that immunomodulation taking place upon SLIT may have a general tolerance-inducing bystander effect, also on other off-target allergens. However, a pilot study of bystander effects of a 4-month treatment with *P. pratense* SLIT tablet Grastek[®] could not prove any effect on birch-induced allergic rhinitis in adults with both birch and grass pollen allergy.²⁶

While investigating the effect of GRAZAX[®] treatment on circulating mediators in plasma samples, GRAZAX[®]-treated subjects exhibited maintained higher levels of CXCL10 at the end of treatment, and both CXCL10 and CXCL11 were sustained at elevated levels at the follow-up 2 years later, relative to the placebo group. The IFN- γ -induced chemokines CXCL10 and CXCL11 are produced by epithelial cells,²⁷ macrophages,²⁸ and dendritic cells²⁹ among others and play important roles in the recruitment and activation of different

immune cells which counteract eg Th2-responses. In contrast to the untreated individuals, where the Th2-skewing of the allergic immune system may have repressed the Th1-response, treatment with GRAZAX[®] seemed to maintain high levels of the Th1-associated chemokines. This was further affirmed by the strong reduction in the Th2/Th1-ratios of cytokine levels after 3 years with treatment. Our findings imply that SLIT treatment modulates immune cells not only toward less Th2-related inflammation locally (in saliva) but also systemically. Examining the ratio of Th2 immune responses over the Th1-response constitutes a way to study how proportions of the respective immune responses vary over time. Previous studies by our laboratory have linked allergy development to altered Th2/Th1-ratios of allergy-associated chemokine levels.^{20-22,30} In this study, the ratios of IL-5 and IL-13 over IFN- γ levels in timothy grass pollen-stimulated supernatants were significantly lower in treated subjects. Intriguingly, it was recently shown that a Th2-associated memory T-cell population positive for IL-5 expression was attenuated in response to a 1-year-long SLIT treatment against house dust mites in subjects with allergic rhinitis,³¹ altogether pointing at a reduction in the Th2- over the Th1-response, and hence less allergic inflammation in the circulation. In addition, we showed that the IL-5/IFN- γ ratio was also strongly inversely correlated with the levels of Phl p 1-specific IgA, suggesting that the induction of tolerance against timothy grass pollen in these children is related to a reduction in Th2-immunity.

In conclusion, *Phleum pratense* SLIT augmented the levels of Phl p 1-specific IgA in saliva and elevated circulating levels of specific IgG₄, whereas levels of Th1-associated chemokines CXCL10 and CXCL11 in children with timothy grass pollen allergy were maintained elevated compared to the placebo group. Furthermore, lower IL5/IL13 vs. IFN- γ ratios in allergen-stimulated cells imply alterations of the prevailing Th2-skewing in the immune system toward a balance between Th2- and Th1-responses, which was also reflected in less symptoms of ARC in treated subjects. Altogether, both local and systemic effects of a 3-year treatment with SLIT modulated the immune response of timothy grass pollen allergic children toward a tolerant state, which remained 2 years after treatment cessation.

ACKNOWLEDGMENTS

We would like to thank Ulla Nyström and Kicki Helander for their brilliant and enthusiastic work guiding the children through the study and all the sampling procedures, and Ammi Fornander and Camilla Janefjord for their excellent technical assistance. Additionally, we thank Martina Abenius for valuable advice on the bead coupling procedure. Also, we would like to thank ALK Nordic A/S for the opportunity to perform this sub-study within the GAP study.

CONFLICT OF INTEREST

J. Alm, J. Huoman, and M. Jenmalm have received honoraria from ALK for lectures.

AUTHOR CONTRIBUTIONS

MCJ and LJN designed the study. JA and LJN were responsible for clinical evaluation of the children. JH, GP, and AP performed experimental work. JH performed statistical analyses and presented the data. All authors interpreted and discussed the results. JH drafted the manuscript. All authors contributed to and approved the final draft for publication.

ORCID

Johanna Huoman  <https://orcid.org/0000-0003-2509-2418>

Lennart J. Nilsson  <https://orcid.org/0000-0002-5680-6367>

Maria C. Jenmalm  <https://orcid.org/0000-0002-2117-5366>

REFERENCES

- Asher MI, Montefort S, Björkstén B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet (London, England)*. 2006;368(9537):733-743.
- Blaiss MS, Hammerby E, Robinson S, Kennedy-Martin T, Buchs S. The burden of allergic rhinitis and allergic rhinoconjunctivitis on adolescents: A literature review. *Ann Allergy Asthma Immunol*. 2018;121(1):43-52.
- Porcaro F, Corsello G, Pajno G. SLIT's prevention of the allergic march. *Curr Allergy Asthma Rep*. 2018;18(5):31.
- Valovirta E, Petersen TH, Piotrowska T, et al. Results from the 5-year SQ grass sublingual immunotherapy tablet asthma prevention (GAP) trial in children with grass pollen allergy. *J Allergy Clin Immunol*. 2018;141(2):529-538.
- Hoffmann HJ, Valovirta E, Pfaar O, et al. Novel approaches and perspectives in allergen immunotherapy. *Allergy*. 2017;72(7):1022-1034.
- Shamji MH, Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. *J Allergy Clin Immunol*. 2017;140(6):1485-1498.
- Durham SR, Penagos M. Sublingual or subcutaneous immunotherapy for allergic rhinitis? *J Allergy Clin Immunol*. 2016;137(2):339-349.
- Scadding GW, Shamji MH, Jacobson MR, et al. Sublingual grass pollen immunotherapy is associated with increases in sublingual Foxp3-expressing cells and elevated allergen-specific immunoglobulin G4, immunoglobulin A and serum inhibitory activity for immunoglobulin E-facilitated allergen binding to B cells. *Clin Exp Allergy*. 2010;40(4):598-606.
- Varona R, Ramos T, Escribese MM, et al. Persistent regulatory T cell response 2 years after 3 years of grass tablet SLIT: links to reduced eosinophil counts, sIgE levels and clinical benefit. *Allergy*. 2018;74(2):349-360. [Epub ahead of print].
- Jacobsen L, Niggemann B, Dreborg S, 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(8):943-948.
- Möller C, Dreborg S, Ferdousi HA, et al. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol*. 2002;109(2):251-256.
- Niggemann B, Jacobsen L, Dreborg S, et al. Five-year follow-up on the PAT study: specific immunotherapy and long-term prevention of asthma in children. *Allergy*. 2006;61(7):855-859.
- Valovirta E, Berstad A, de Blic J, et al. Design and recruitment for the GAP trial, investigating the preventive effect on asthma development of an SQ-standardized grass allergy immunotherapy tablet in children with grass pollen-induced allergic rhinoconjunctivitis. *Clin Ther*. 2011;33(10):1537-1546.
- Macpherson AJ, McCoy KD, Johansen F, Brandtzaeg P. The immune geography of IgA induction and function. *Mucosal Immunol*. 2008;1(1):11-22.
- Novak N, Haberstick J, Bieber T, Allam J-PP. The immune privilege of the oral mucosa. *Trends Mol Med*. 2008;14(5):191-198.
- Sandin A, Björkstén B, Böttcher MF, Englund E, Jenmalm MC, Bråbäck L. High salivary secretory IgA antibody levels are associated with less late-onset wheezing in IgE-sensitized infants. *Pediatr Allergy Immunol*. 2011;22(5):477-481.
- Fagerås M, Tomićić S, Voor T, Björkstén B, Jenmalm MC. Slow salivary secretory IgA maturation may relate to low microbial pressure and allergic symptoms in sensitized children. *Pediatr Res*. 2011;70(6):572-577.
- Scaparrotta A, Verini M, Consilvio NP, et al. Sensitization to timothy grass pollen allergenic molecules in children. *Multidiscip Respir Med*. 2013;8(1):17.
- Cipriani F, Mastrorilli C, Tripodi S, et al. Diagnostic relevance of IgE sensitization profiles to eight recombinant Phleum pratense molecules. *Allergy*. 2018;73(3):673-682.
- Abelius MS, Ernerudh J, Berg G, Matthiesen L, Nilsson LJ, Jenmalm MC. High cord blood levels of the T-helper 2-associated chemokines CCL17 and CCL22 precede allergy development during the first 6 years of life. *Pediatr Res*. 2011;70(5):495-500.
- Abelius MS, Lempinen E, Lindblad K, et al. Th2-like chemokine levels are increased in allergic children and influenced by maternal immunity during pregnancy. *Pediatr Allergy Immunol*. 2014;25(4):387-393.
- Sandberg M, Frykman A, Ernerudh J, et al. Cord blood cytokines and chemokines and development of allergic disease. *Pediatr Allergy Immunol*. 2009;20(6):519-527.
- Liu Y, Xing Z, Wang J, Geng C. Salivary immunoglobulin A, E, and G4 levels specific to dermatophagoides pteronyssinus in allergic rhinitis patients treated with subcutaneous immunotherapy. *Am J Rhinol Allergy*. 2018;32(6):458-464.
- van de Veen W, Akdis M. Role of IgG4 in IgE-mediated allergic responses. *J Allergy Clin Immunol*. 2016;138(5):1434-1435.
- Suárez-Fuero A, Ramos T, Galán A, et al. Grass tablet sublingual immunotherapy downregulates the TH2 cytokine response followed by regulatory T-cell generation. *J Allergy Clin Immunol*. 2014;133(1):130-138.
- Ellis AK, Tenn MW, Steacy LM, et al. Lack of effect of Grastek® on birch pollen-induced allergic rhinoconjunctivitis in the Environmental Exposure Unit. *Ann Allergy Asthma Immunol*. 2018;120(5):495-503.
- Fenwick PS, Macedo P, Kilty IC, Barnes PJ, Donnelly LE. Effect of JAK inhibitors on release of CXCL9, CXCL10 and CXCL11 from Human Airway Epithelial Cells. *PLoS ONE*. 2015;10(6):e0128757.
- Karta MR, Gavala ML, Curran CS, et al. LPS modulates rhinovirus-induced chemokine secretion in monocytes and macrophages. *Am J Respir Cell Mol Biol*. 2014;51(1):125-134.
- Lebre MC, Burwell T, Vieira PL, et al. Differential expression of inflammatory chemokines by Th1- and Th2-cell promoting dendritic cells: A role for different mature dendritic cell populations in attracting appropriate effector cells to peripheral sites of inflammation. *Immunol Cell Biol*. 2005;83(5):525-535.
- Abrahamsson TR, Sandberg Abenius M, Forsberg A, Björkstén B, Jenmalm MC. A Th1/Th2-associated chemokine imbalance during infancy in children developing eczema, wheeze and sensitization. *Clin Exp Allergy*. 2011;41(12):1729-1739.

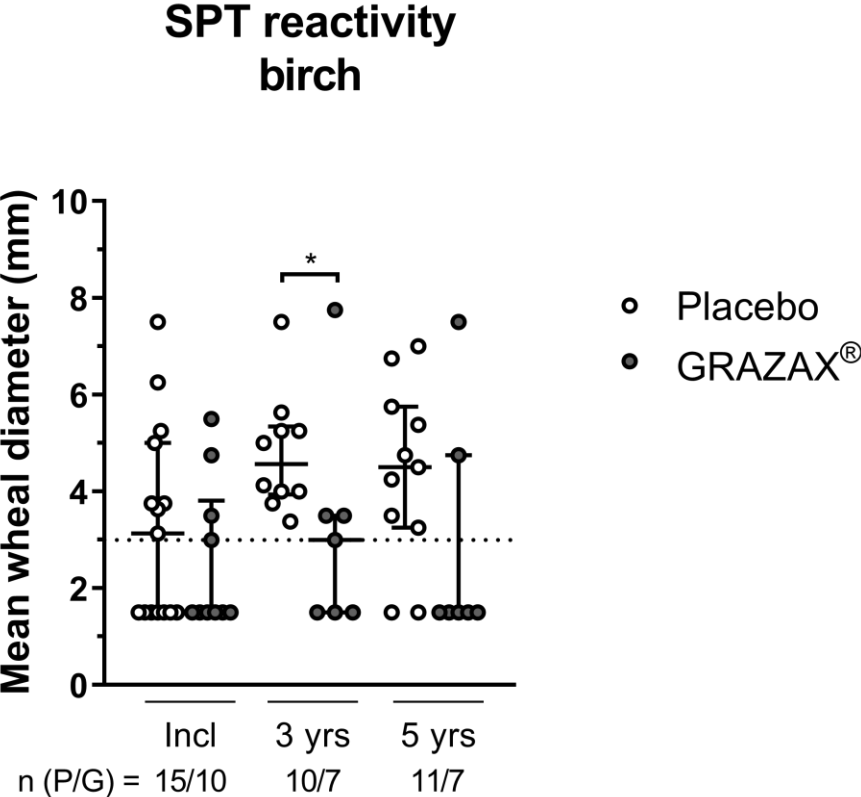
31. Ihara F, Sakurai D, Yonekura S, et al. Identification of specifically reduced Th2 cell subsets in allergic rhinitis patients after sublingual immunotherapy. *Allergy*. 2018;73(9):1823-1832.

SUPPORTING INFORMATION

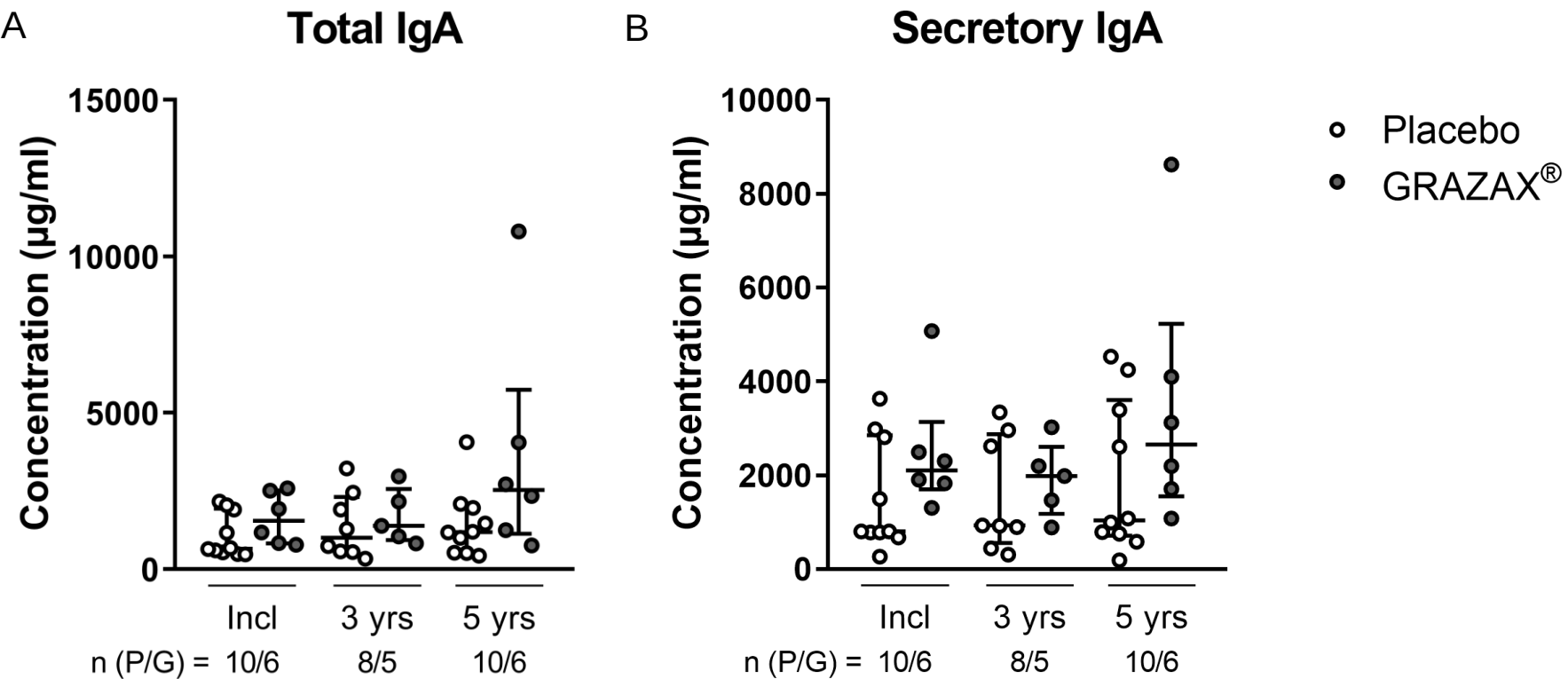
Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Huoman J, Papapavlou G, Pap A, Alm J, Nilsson LJ, Jenmalm MC. Sublingual immunotherapy alters salivary IgA and systemic immune mediators in timothy allergic children. *Pediatr Allergy Immunol*. 2019;30:522–530. <https://doi.org/10.1111/pai.13047>

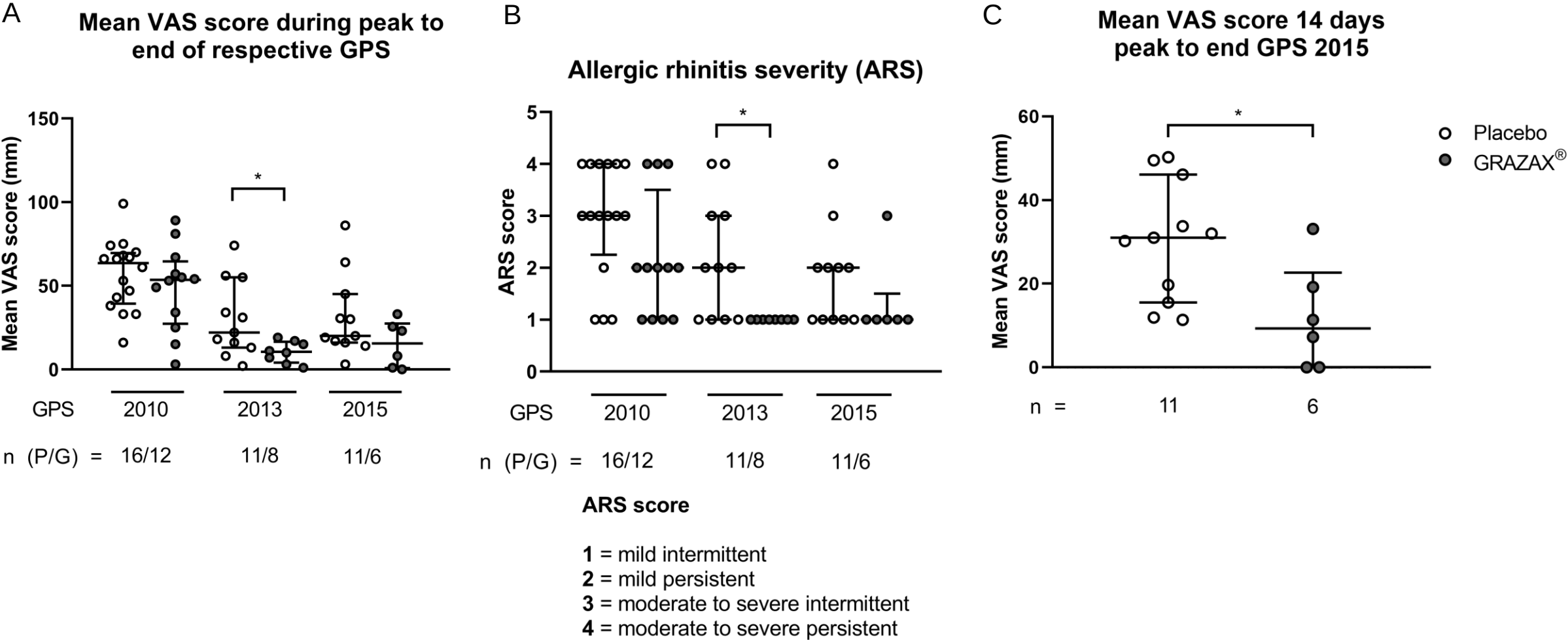
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



Appendix 1

Details on Methods section

Methods

The present study utilized samples from the international multicenter GRAZAX[®] Asthma Prevention (GAP) study, a randomized, double-blind, placebo-controlled intervention study aiming at investigating possible asthma preventive effects of grass pollen-specific immunotherapy in children with current grass pollen-induced allergic rhinoconjunctivitis (ARC).¹ Children between 5-12 years of age with grass pollen-induced ARC, as evaluated and defined by specific skin prick test positivity (wheal diameter ≥ 3 mm) towards timothy grass (*Phleum pratense*) and positive *P. pratense* specific IgE-levels (sIgE ≥ 0.7 kU/L) in serum, were included in the study. Exclusion criteria comprised seasonal and/or perennial ARC caused by allergens other than timothy grass, previous history, suspicion or diagnosis of asthma, as well as previous immunotherapy treatment with cross-reacting allergens or grass pollen allergen. The active treatment consisted of lyophilized SQ sublingual tablets containing standardized timothy grass pollen allergen extracts from *Phleum pratense* (GRAZAX[®], ALK, Hørsholm, Denmark), whereas the placebo treatment consisted of a sublingual tablet without grass pollen extract, non-distinguishable from the active treatment in appearance. Upon inclusion in the study, the participants were administering the tablet sublingually once daily for three consecutive years, followed by a two-year follow-up period. The study subjects were followed-up for a total of 15 visits during the five-year study period, which included e.g. investigations of ARC symptoms and medication use, development of asthma, physical examinations as well as investigations of immunological markers such as grass pollen-specific IgE, IgG₄ and skin prick tests (SPTs).¹ The SPTs were consistently performed by the same research nurse at both sites. Additionally, for the present study, blood and saliva samples were collected at inclusion in the study (visit 3 – randomization visit, after grass pollen season (GPS) 2010), after three years of treatment (visit 10 – after GPS 2013), and two years after the treatment ended (visit 15 - after GPS 2015). Furthermore, information about ARC symptoms, such as visual analogue scale (VAS) data on allergic rhinitis symptoms and an allergic rhinitis severity (ARS) score were collected. Two different VAS scale measurements were included. On one hand, incident VAS data from the visits 2, 9 and 14 in peak to end of the respective GPS for 2010, 2013 and 2015, were recorded as an answer to the question “How has your hay fever been the last week?”. On the other hand, VAS data collected in a diary from 14 consecutive days in peak to end of the GPS 2015, as an answer to the question “How has your hay fever been today?” were included. The ARS score consists of four categories: 1 = mild intermittent, 2 = mild

36 persistent, 3 = moderate to severe intermittent and 4 = moderate to severe persistent as
37 recorded by a research nurse at the visits in peak to end of the respective GPS in 2010,
38 2013 and 2015. Samples utilized in this sub-study originate from two study sites in Sweden;
39 Linköping (Allergy center, Linköping University hospital, Linköping, Sweden) and Stockholm
40 (Sachs' children and youth hospital, Södersjukhuset, Stockholm, Sweden). From the total
41 812 individuals included in the entire study², in total 28 children were included from the
42 mentioned study sites, of whom 17 completed the study, and samples were available from 25
43 unique individuals. Informed consent was obtained from parents or legal guardians of the
44 children. The study was approved by the Regional Ethics Committee for Human Research in
45 Linköping, Sweden (Dnr 2010/430, 2009/551, 2009-0111235-12, 151:2010/48756).

47 ***Blood sample preparation***

48 **Cell separation and allergen stimulations**

49 Peripheral blood samples were drawn into sodium heparin tubes (KIMA, Arzergrande, Italy),
50 and subsequently, peripheral blood mononuclear cells (PBMCs) were isolated from whole
51 blood as previously described.³ Thereafter, 1×10^6 cells were stimulated with 10 kSU/ml
52 timothy grass allergen (Aquagen *Phleum pratense*, ALK, Hørsholm, Denmark) and 10
53 kSU/ml birch allergen (Aquagen *Betula verrucosa*, ALK) separately, for 24h and 6 days at
54 37°C (5% CO₂). Unstimulated negative controls were included for both time points, whereas
55 the positive control (2 µg/ml PHA, Sigma Aldrich, St. Louis, MI, USA) was only added to the
56 24h time point. At the end of incubation, supernatants were collected upon centrifuging the
57 samples at 1400 rpm for 10 min at RT, and subsequently frozen at -70 °C until further use.

58 ***Blood sample analyses***

59 **Determination of IFN-γ, IL-5, IL-10, IL-13, CCL17 and CXCL10 by multiplex bead assay** 60 **in allergen stimulated PBMC supernatants**

61 Allergy related cytokines (IFN-γ, IL-5, IL-10 and IL-13) and chemokines (CCL17 and
62 CXCL10) were analyzed in supernatants of allergen stimulated PBMCs by means of a
63 previously developed in-house 6-plex multiplex bead assay.⁴ The protocol was followed with
64 a few exceptions. The 8-point standard curve was prepared from recombinant human
65 proteins in steps of 1:3 in AIM-V (Gibco, NY, USA) + 20 µM β-mercaptoethanol (Gibco,
66 Paisley, UK) for the respective analytes to the following concentration ranges: IFN-γ (10 000-
67 4.57 pg/ml, R&D Systems), IL-5 (3 333-1.52 pg/ml, BD Pharmingen), IL-10 (833.33-0.38
68 pg/ml, Sanquin), IL-13 (30 000-13.7 pg/ml, R&D Systems), CCL17 (466.66-0.21 pg/ml, R&D
69 Systems) and CXCL10 (4 000-1.83 pg/ml, R&D systems). The AIM-V + β-mercaptoethanol

buffer was also used as a blank. Identical polyclonal biotinylated detection antibodies were used but in different end concentrations; 500 ng/ml IFN- γ (clone: 4S.B3, BD Pharmingen), 500 ng/ml IL-5 (clone: JES1-5A10, BD Pharmingen), 100 ng/ml IL-10 (clone: M191004, Sanquin), 200 ng/ml IL-13 (clone: B69-2, Sanquin), 500 ng/ml CCL17 (Cat.no: BAF364, R&D systems) and 1000 ng/ml CXCL10 (clone: 6D4/D6/G2, BD Pharmingen). The plate was analyzed in a Luminex 200 instrument (Luminex Corp., Austin, TX, USA), utilizing the Luminex Xponent (Version 3.1.871.0) and Masterplex 2000 (Version 2.0.0.68) softwares for result extraction and evaluation, respectively. Detection limits; IL-10: 0.38 pg/ml, IFN- γ : 13.72 pg/ml, CXCL10: 5.49 pg/ml, CCL17: 0.64 pg/ml, IL-13: 41.15 pg/ml and IL-5: 1.52 pg/ml. Inter assay variation: 16-24%.

ELISA for detection of IL-10 in allergen stimulated PBMC supernatants

A commercially available ELISA antibody pair (PeliPair™ human IL-10 ELISA reagent set, Sanquin) was used to investigate IL-10 levels in the 24h supernatant samples except the PHA control, as previously described.⁵ The protocol was followed with the following exceptions: During the coating step, the plate was incubated in room temperature (RT) for 1h while shaking, and then over night. A standard curve of 7 points in steps of 1:2 was set in the range of 150-2.35 pg/ml. The enzyme conjugate SA-poly-HRP (Art.no: M2032, Sanquin) was added in a concentration of 100 pg/ml. Detection limit: 4.7 pg/ml. Inter assay variation: 10-20%.

4-plex Luminex for detection of CCL17, CCL22, CXCL10 and CXCL11 in plasma samples

Utilizing a previously described in-house multiplex bead assay⁶, the chemokines CCL17, CCL22, CXCL10 and CXCL11 were analyzed as a four-plex assay in plasma samples. Standard ranges: CCL17, CCL22 and CXCL10 (1400-1.9 pg/ml), CXCL11 (4000-5.5 pg/ml) in 7 standard points 1:3 dilution in AIM-V (Gibco, also used as a blank). The set plate was incubated for 1h at RT on a plate shaker in darkness and afterwards overnight at 4°C on a shaker. A final washing step was performed followed by the addition of 75 μ l washing buffer in each well. The plate was shaken for 5-10 minutes at RT in darkness prior to analysis using a Luminex 200 instrument (Luminex Corp. Austin, TX, USA), and Masterplex 2000 software (V2.0.0.68) for evaluation of the results. Blank values were subtracted from all readings and the CV limit was set to 15. Detection limits; CCL17: 0.64 pg/ml, CCL22 and CXCL10: 1.92 pg/ml, CXCL11: 16.5 pg/ml. Inter assay variation: 2-31%.

In-house ELISA for detection of CCL18 in plasma

A commercial ELISA CCL18 DuoSet ELISA kit (Cat.no: DY394-05, R&D Systems, Minneapolis, MN, USA) was utilized to investigate CCL18 levels in plasma samples, as previously described.⁷ With the following exceptions, the protocol was followed; plates were coated with 4 µg/ml monoclonal anti-human CCL18/PARC, the standard curve consisting of Recombinant Human CCL18/PARC Protein was diluted in steps of 1:2 into 7 standard points (250-3.9 pg/ml) and the human CCL18/PARC Biotinylated Antibody was added to the plates in a final concentration of 100 ng/ml. The detection limit was 15.6 pg/ml. Inter assay variation: 12-18%.

Saliva sample preparation

Collection and heat-inactivation

Saliva samples were collected by suction (passive drooling), and subsequently stored at -20°C. Prior to further analyses, the saliva samples were thawed and heat-inactivated at 56°C in a water bath for 30 min. Thereafter, the samples were centrifuged at 5000 g for 15 min, and supernatants were transferred to new tubes before subsequent downstream analyses or storage in -70°C.

Saliva sample analyses

In-house ELISA for detection of total IgA in saliva

A Costar 3690 96-well plate was coated with rabbit anti-human IgA (DAKO, A-0262, Glostrup, Denmark) diluted 1:1000 in PBS (Medicago, Uppsala, Sweden), and incubated for 2h in a humid chamber at 37°C. Thereafter, an additional 3h incubation at 4°C followed, prior to washing the plate four times in an automated washer (Tecan Hydroflex, Tecan Austria GmbH, Grödig, Austria). Subsequently, the plate was blocked with PBS (Medicago) + 0.5% BSA (Sigma Aldrich), and incubated for 2h at 37°C in a humid chamber, followed by an overnight incubation at 4°C. On the next day, a seven-point standard curve was diluted in steps of 1:2 (2000-31.25 ng/ml, Human IgA, I1010, Sigma Aldrich) in PBS-T (Medicago) + 0.5% BSA (Sigma Aldrich). Plate wash followed, as previously described and the standard curve was added to the plate along with diluted samples, controls and blanks (PBS-T + 0.5% BSA) in duplicates, and the plate was incubated at 37°C in a humid chamber for 1h. An anti-human peroxidase conjugated detection antibody (Art.no: A0295, Sigma Aldrich) was diluted 1:50 000 in PBS-T + 0.5% BSA, and added to the plate upon washing, and incubated for another hour in the humid chamber at 37°C. Upon repeated washing, the substrate 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma Aldrich) was added to the plate, and incubated for 30 min while standing in RT. The enzymatic reaction was stopped using 1.8 M H₂SO₄ (Scharlab,

Barcelona, Spain), and was thereafter immediately read in an ELISA reader (Tecan Sunrise, Tecan Austria GmbH, Grödig, Austria) at 405 nm (reference wavelength 540 nm). Detection limit: 62.5 ng/ml. Inter assay variation: 2-8%.

In-house ELISA for detection of secretory IgA in saliva

This assay procedure was identical to the above described Total IgA ELISA in saliva, with the following exceptions. In the initial coating step, the plate was coated with a monoclonal anti-human secretory component antibody (IgA, clone: GA-1, Sigma Aldrich) diluted 1:10 000 in PBS (Medicago). The detection antibody used was the very same antibody as above, but it was diluted 1:25 000 in PBS-T + 0.5% BSA instead. Detection limit: 62.5 ng/ml. Inter assay variation: 26-41%.

1-plex Luminex Phl p 1-specific IgA in saliva

For the detection of timothy grass pollen allergen component Phl p 1-specific IgA antibodies in saliva, an in-house single-plex Luminex assay was developed. Firstly, magnetic microbeads (Luminex Corp.) were coupled to 10 µg Phl p 1 allergen (BioMay, Vienna, Austria) per million beads, according to the manufacturer's instructions. The coupling procedure was validated by measuring Phl p 1-specific IgG₁ in a serum sample known to contain these antibodies. Thereafter, saliva samples were thawed at room temperature and centrifuged for 10 min, RT, 7400g. Meanwhile, a standard curve was prepared utilizing a saliva sample from a child with known high levels of total and secretory IgA as a reference standard sample. A seven-point standard curve was prepared (1:2-1:1458 end dilution in well) in steps of 1:3 in PBS-T (Medicago) + 1% BSA (Sigma Aldrich), and samples were diluted 1:10-1:250 to avoid matrix effects of the samples. Samples, standard curve and blanks (PBS-T + 1% BSA) were added in duplicates to a pre-wetted black flat-bottom micro plate (Greiner Bio-One, Kremsmünster, Austria). Thereafter, an amount of 2000 Phl p 1-coupled magnetic beads per 50 µl were added to the plate upon thorough vortexing and sonication. The plate was incubated for 1h in RT on a plate shaker (800 rpm) followed by an over-night incubation at 4°C while shaking (600 rpm). The following day, the plate was taken out in RT and allowed to shake for a while (800 rpm). Meanwhile, a biotinylated goat-anti human IgA secondary antibody (Art.no: PA-86062, Thermo Fischer) was diluted in PBS-T + 1% BSA to a concentration of 250 ng/ml. The plate was washed four times in PBS-T + 1% BSA, utilizing an automated magnetic washer (Tecan Hydroflex, Tecan Austria GmbH, Grödig, Austria). Subsequently, the detection antibody was added to each well, and the plate was incubated for 1h at RT while shaking (800 rpm). The plate was washed as described above, and 1 µg/ml SA-PE (Life Technologies corporation, Eugene, Oregon, USA) in PBS-T + 1% BSA, was added to the plate followed by a 30 min incubation while shaking in RT (800

rpm). The washing step was repeated as above, and finally the beads were re-suspended in sheath fluid, and let shake for a while in RT prior to analysis on a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA), and evaluation using the Masterplex 2000 software (version 2.0.0.68). On a seven-point scale in arbitrary units (AUs) starting from 1000, the calculated detection limit corresponded to 0.69 AUs (half the concentration of the lowest standard point). Inter assay variation: 9%.

Skin prick test evaluation, IgG₄ and IgE level analysis

SPTs were performed in duplicate on the volar aspects of the forearms with standardized birch and timothy allergen extracts (Soluprick, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/ml) was used as a positive control and albumin diluent was included as a negative control. The test was regarded as positive if the mean diameter was >3 mm. The levels of IgE (range 0.7-800 kU/L) and IgG₄ antibodies (range 0.07-30 mg/L) to timothy grass allergen were analyzed in the ALK laboratory in Hørsholm using Phadia250 ImmunoCAP according to the recommendations by the manufacturer (Thermo Fisher Scientific, Uppsala, Sweden).

Statistics

Unless otherwise stated, samples were considered appropriate for analysis with a CV of less than 15. Undetectable values were given half of the cut-off limit for the respective analyte. Comparisons between the intervention groups were statistically evaluated using Mann Whitney *U*-test, due to the non-normal distribution of most of the variables. To study the effect of treatment over time on Phl p 1-specific IgA antibody levels, a Friedman test with Dunn's multiple comparisons test was applied separately in the treatment groups for those individuals who had samples from all three time points (GRAZAX[®] n = 5, Placebo n = 8). In cytokine and chemokine data originating from PBMC allergen stimulations, the respective unstimulated controls were subtracted from the stimulated sample values prior to statistical analysis. Correlation analyses of clinical and immunological variables were performed using the non-parametric Spearman's rank test. Analyses were performed in GraphPad Prism 7 (Version 7.04, GraphPad Software Inc., La Jolla, CA, USA) and IBM[®] SPSS[®] Statistics (Version 25.0.0.1, IBM Corp., Armonk, NY, USA).

Supplementary figure legends

Supplementary Figure 1. Mean wheal diameter (in mm) upon SPT with birch allergen. All measurements were performed at inclusion, after 3 years of treatment and 2 years after treatment ending (5 years from inclusion time point). White circles indicate placebo treatment and grey circles GRAZAX[®] treatment. The dotted line indicates the cut-off for SPT-positivity. The numbers of individuals analyzed are indicated below each time point as n (P=Placebo/G=GRAZAX[®]). The data are illustrated as medians with interquartile ranges. *p < 0.05, Mann-Whitney U test.

Supplementary Figure 2. Total (A) and secretory (B) levels of salivary IgA, as determined by in-house ELISA measurements. All measurements were performed at inclusion, after 3 years of treatment and 2 years after treatment ending (5 years from inclusion time point). White circles indicate placebo treatment and grey circles GRAZAX[®] treatment. The numbers of individuals analyzed are indicated below each time point as n (P=Placebo/G=GRAZAX[®]). The data are illustrated as medians with interquartile ranges. Mann-Whitney U tests revealed no significant differences.

Supplementary Figure 3. Visual Analogue Scale (VAS) reported for allergic rhinitis symptoms (in mm) in A. as an answer to the question “How has your hay fever been the last week?” represented as incident data from peak to end of the respective grass pollen season (GPS) in 2010, 2013 and 2015. These timepoints are in conjunction to the sampling time points at inclusion, after three years of treatment and two years after treatment end. B illustrates allergic rhinitis severity (ARS) score in relation to intervention group belonging from peak to end of the respective GPS in 2010, 2013 and 2015. The score consists of four categories: 1 = mild intermittent, 2 = mild persistent, 3 = moderate to severe intermittent and 4 = moderate to severe persistent. In C. VAS data are reported as a mean of 14 day data (in mm) as an answer to the question “How has you hay fever been today?” in peak to end of the grass pollen season (GPS) of 2015. White circles indicate placebo treatment and grey circles GRAZAX[®] treatment. The numbers of individuals analyzed are indicated below each time point as n (P=Placebo/G=GRAZAX[®]). The data are illustrated as medians with interquartile ranges. *p < 0.05, Mann-Whitney U test.

Supplementary Table I. Cytokine and chemokine data from Luminex and ELISA analyses in

<i>Plasma (pg/ml)</i>			
	Inclusion	3 years	5 years
CCL17	14.2 (7.4-24.0)	10.5 (5.4-20.2)	16.5 (10.9-23.5)
CCL18[†]	57355 (47014-81990)	46207 (32693-60844)	35257 (27873-52404)
CCL22	75.6 (55.2-94.7)	90.4 (58.3-103.6)	66.5 (30.4-87.7)
<i>PBMC supernatants (pg/ml)</i>			
Grass stimulations[‡]	Inclusion	3 years	5 years
CCL17	64.3 (6.5-229.4)	110.9 (26.8-269.2)	65.8 (26.3-164.8)
CXCL10	180.0 (2.8-832.1)	2.8 (2.8-1409.0)	853.8 (22.3-1668.0)
IFNγ	6.9 (6.9-12.6)	6.9 (6.9-6.9)	6.9 (6.9-71.2)
IL-5	27.6 (1.6-106.3)	30.8 (6.0-72.8)	15.2 (2.3-38.0)
IL-13	314.8 (20.6-1541.0)	427.0 (20.6-1055.0)	323.9 (143.9-998.1)
IL-10 24h[†]	2.4 (2.4-4.9)	2.4 (2.4-10.3)	2.4 (2.4-2.4)
IL-10	1.9 (0.2-7.2)	1.4 (0.2-4.6)	2.5 (1.0-4.2)
Birch stimulations[‡]	Inclusion	3 years	5 years
CCL17	711.7 (248.7-1693.0)	789.1 (166.6-1391.0)	752.8 (187.7-1069.0)
CXCL10	2.8 (2.8-647.2)	238.3 (2.8-1317.0)	63.5 (2.8-1093.0)
IFNγ	37.6 (6.9-67.0)	6.9 (6.9-17.3)	37.3 (6.9-64.8)
IL-5	442.2 (188.7-879.1)	364.2 (215.4-696.7)	491.0 (268.9-746.0)
IL-13	6500 (2669-14974)	3721 (1326-8566)	6242 (2011-9806)
IL-10 24h[†]	5.3 (2.4-9.3)	2.4 (2.4-9.7)	3.8 (2.4-9.9)
IL-10	27.9 (8.4-38.7)	22.5 (11.2-41.2)	16.5 (7.8-33.7)

plasma as well as in birch and grass stimulated PBMC supernatants.

Supernatants were harvested at 6 days after beginning of experiment, except for a number of samples harvested at 24h for analyses of monocyte derived IL-10 levels. All samples were analyzed by means of Luminex methodology, unless marked with [†] indicating analyses by means of ELISA. All measurements were performed at inclusion, after 3 years of treatment and 2 years after treatment ending (5 years from inclusion time point). Measured concentrations (pg/ml) are presented as medians with interquartile ranges (Q1-Q3), where the negative controls for the respective allergen stimulations have been subtracted.

[†] Analyses performed by means of ELISA

[‡] The respective medium controls have been subtracted from the measured analytes in data from the stimulation experiments.

248 **References**

- 249 1. Valovirta E, Berstad AK, de Blic J, et al. Design and recruitment for the GAP trial, investigating
250 the preventive effect on asthma development of an SQ-standardized grass allergy
251 immunotherapy tablet in children with grass pollen-induced allergic rhinoconjunctivitis. *Clin*
252 *Ther.* 2011;33:1537-1546.
- 253 2. Valovirta E, Petersen TH, Piotrowska T, et al. Results from the 5-year SQ grass sublingual
254 immunotherapy tablet asthma prevention (GAP) trial in children with grass pollen allergy. *J*
255 *Allergy Clin Immunol.* 2018;141:529.
- 256 3. Forsberg A, Abrahamsson TR, Björkstén B, Jenmalm MC. Pre- and postnatal administration of
257 *Lactobacillus reuteri* decreases TLR2 responses in infants. *Clin Transl Allergy.* 2014;4:21.
- 258 4. Forsberg A, Abrahamsson TR, Björkstén B, Jenmalm MC. Pre- and post-natal *Lactobacillus*
259 *reuteri* supplementation decreases allergen responsiveness in infancy. *Clin Exp Allergy.*
260 2013;43:434-442.
- 261 5. Böttcher MF, Bjurström J, Mai X-MM, Nilsson L, Jenmalm MC. Allergen-induced cytokine
262 secretion in atopic and non-atopic asthmatic children. *Pediatr Allergy Immunol.* 2003;14:345-
263 350.
- 264 6. Abrahamsson TR, Sandberg Abenius M, Forsberg A, Björkstén B, Jenmalm MC. A Th1/Th2-
265 associated chemokine imbalance during infancy in children developing eczema, wheeze and
266 sensitization. *Clin Exp Allergy.* 2011;41:1729-1739.
- 267 7. Sandberg M, Frykman A, Ernerudh J, et al. Cord blood cytokines and chemokines and
268 development of allergic disease. *Pediatr Allergy Immunol.* 2009;20:519-527.

269