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ORIGINAL ARTICLE

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Exploring novel systemic biomarker approaches in grass-pollen sublingual immunotherapy using omics

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Abstract

Background: Sublingual allergen-specific immunotherapy (SLIT) intervention improves the control of grass pollen allergy by maintaining allergen tolerance after cessation. Despite its widespread use, little is known about systemic effects and kinetics associated to SLIT, as well as the influence of the patient sensitization phenotype (Mono- or Poly-sensitized). In this quest, omics sciences could help to gain new insights to understand SLIT effects.

Methods: 47 grass-pollen-allergic patients were enrolled in a double-blind, placebo-controlled, multicenter trial using GRAZAX® during 2 years. Immunological assays (sIgE, sIgG4, and ISAC) were carried out to 31 patients who finished the trial. Additionally, serum and PBMCs samples were analyzed by metabolomics and transcriptomics, respectively. Based on their sensitization level, 22 patients were allocated in Mono- or Poly-sensitized groups, excluding patients allergic to epithelia. Individuals were compared based on their treatment (Active/Placebo) and sensitization level (Mono/Poly).

Results: Kinetics of serological changes agreed with those previously described. At two years of SLIT, there are scarce systemic changes that could be associated to improvement in systemic inflammation. Poly-sensitized patients presented a higher inflammation at inclusion, while Mono-sensitized patients presented a reduced activity of mast cells and phagocytes as an effect of the treatment.

Conclusions: The most relevant systemic change detected after two years of SLIT was the desensitization of effector cells, which was only detected in Mono-sensitized

Abbreviations: AA, Arachidonic acid; AIT, Allergen immunotherapy; FC, Fold change; GC-MS, Gas chromatography coupled to mass spectrometry; GSEA, Gene Set Enrichment Analysis; IMP, Investigational Medical Product; IPA, Ingenuity Pathway Analysis; LC-MS, Liquid chromatography coupled to mass spectrometry; LPCs, Lysophosphatidylcholine; MS/MS, Tandem Mass Spectrometry; PAF, Platelet Activating Factor; PBMCs, Peripheral blood mononuclear cells; QA, Quality Assurance; QC, Quality control; RT, Retention Time; SLIT, Sublingual allergen-specific Immunotherapy.

Tomas Clive Barker-Tejeda and Raphaelle Bazire have equally contributed

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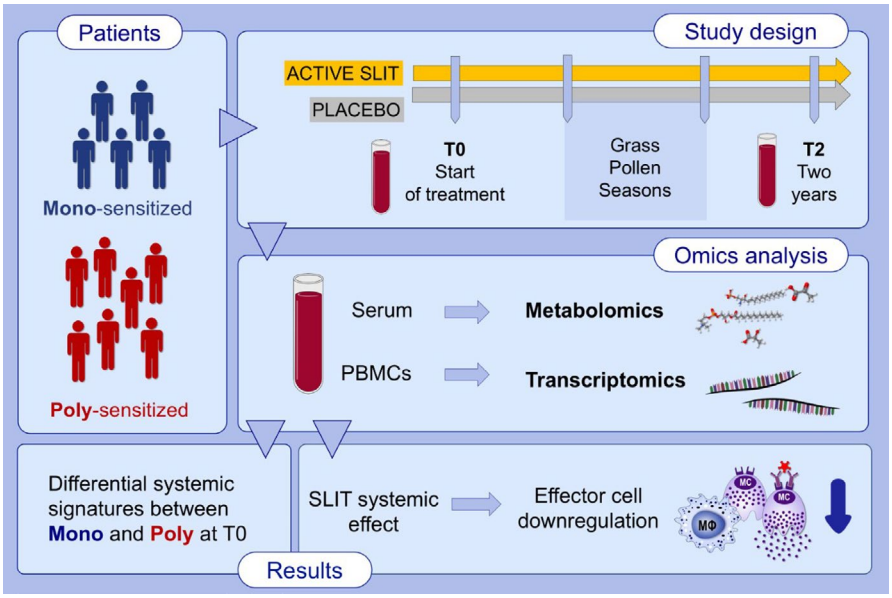
patients. This change may be related to the clinical improvement, as previously reported, and, together with the other results, may explain why clinical effect is lost if SLIT is discontinued at this point.

KEYWORDS

biomarkers, metabolomics, respiratory allergy, sublingual immunotherapy, transcriptomics



GRAPHICAL ABSTRACT

In this study, omics strategies are used to identify mechanisms underlying SLIT in a prospective, double-blind, placebo-controlled study. The systemic effect induced by 2 years of SLIT is mediated by the downregulation of effector cells. Mono- and poly-sensitized patients present differential systemic inflammatory signatures before starting SLIT. Abbreviations: SLIT, sublingual allergen-specific immunotherapy; PBMCs, peripheral blood mononuclear cells.

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1 | INTRODUCTION

Allergen-specific immunotherapy (AIT) constitutes a pivotal pharmacological intervention aiming to control allergic disease. Mechanisms involved include effector cell desensitization, immunoglobulin interference, and T- and B-cell regulation.[1-4]

Allergen immunotherapy is the only intervention that has the potential to modify allergic disease by inducing a long-term effect and preventing evolution to more severe phenotypes. However, this potential is hampered by inadequate AIT use and the lack of adequate biomarkers to monitor intervention effect.[3,5]

One of the main administration routes of AIT is sublingual immunotherapy (SLIT), which has a well-established profile for safety and effectiveness in the treatment of grass pollen allergy.[6]

To date, only a few grass-pollen SLIT products have been developed following a complete clinical development program. In the case of the product analyzed in this study—Grazax®—this program includes over 20 independent clinical trials.[7,8]

One of the most prevalent issues of SLIT is the low compliance rate. There are several factors that contribute to this: (a) the need of administering the therapy daily for three consecutive years; (b) the lack of adequate predictive biomarkers, and (b) the fact that SLIT results in a significant reduction of both allergy symptoms and the use of rescue medication but not in a complete resolution of allergy, and thus it is difficult to evaluate individual clinical benefit. These factors may discourage patients from completing the treatment. However, compliance is essential to achieve the disease-modifying benefit.[9]

In the quest for new biomarkers, we need to understand beforehand the systemic changes induced by SLIT. In a prospective clinical trial with Grazax®, it was observed that both eosinophils count and sIgE levels decrease below starting values only in the third year of intervention.[2] In a similar way, in an asthma prevention five-year study on grass-allergic children,[10] winter asthma symptoms—an indirect measure of bronchial hyperreactivity—were improved in the active group only after three years.

In the last years, system biology approaches, and combined omics approaches, have proved a valuable tool to understand systemic changes associated to severe allergy phenotypes.[11-14] Based on these results, we postulated that similar approaches might shed light on SLIT-associated systemic effects and, more importantly,

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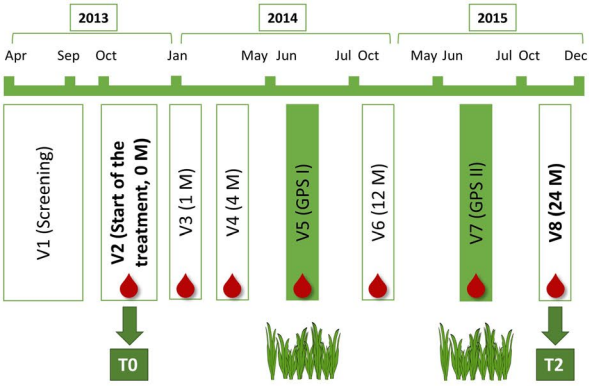


(B)

	Active	Placebo	Total
Mono	4	6	10
Poli	4	8	12
Poli-Epi	6	3	9
Total	14	17	31

→ Excluded from metabolomics and transcriptomics

(C)

		n of patients	
Analysis	Sample	Active	Placebo
Antibody analysis	Serum from all timepoints	14	17
Metabolomics	Serum from T0 and T2	8	14
Transcriptomics	PBMCs from T0 and T2	8	11

II

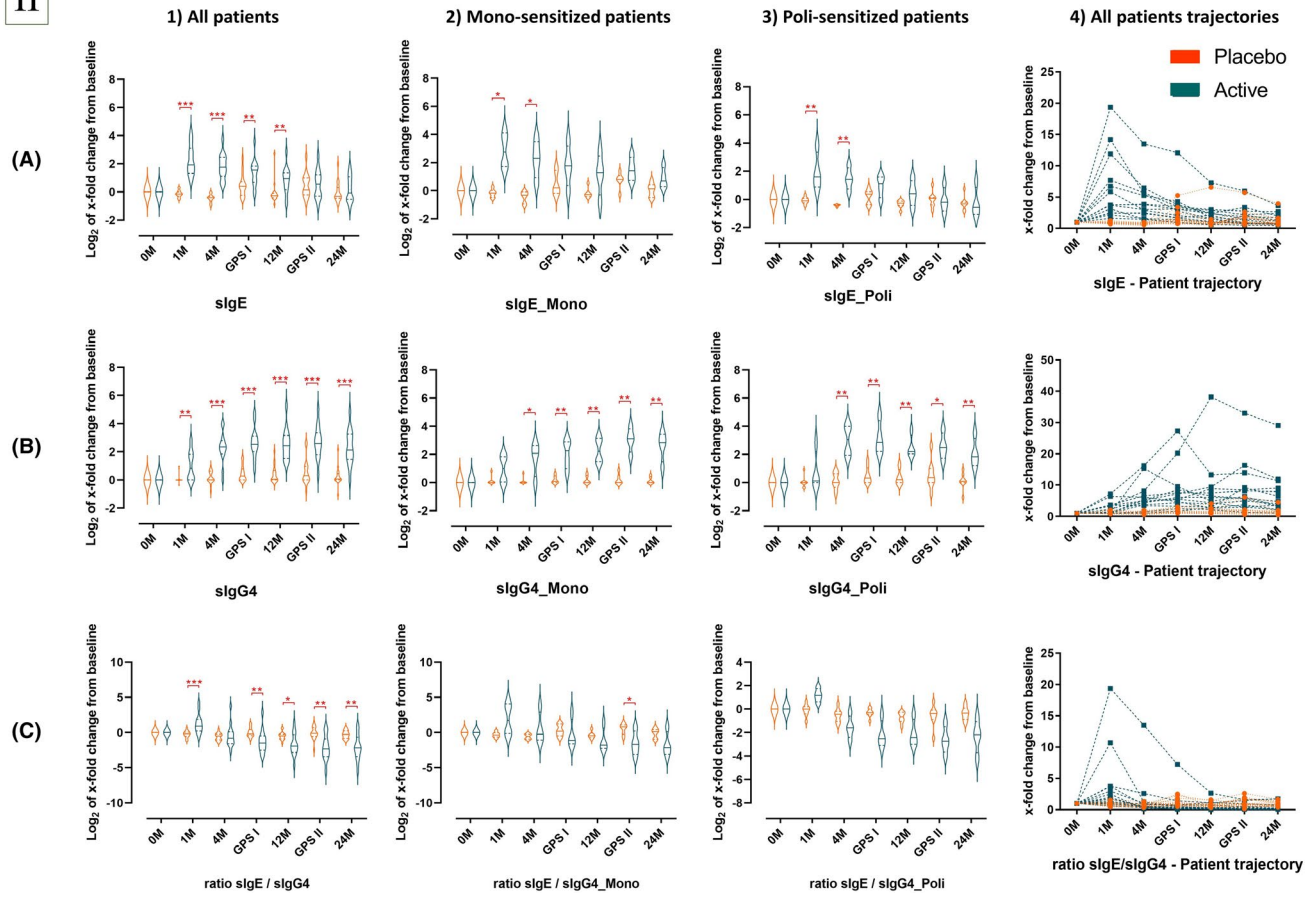


FIGURE 1 I. A, Trial design. V: visit; GPS: grass pollen season; M: month(s); T: time (years). B, Final number of patients in each group according to treatment and sensitization. Mono: Mono-sensitized; Poly: Poly-sensitized; Poly-Epi: Poly-sensitized with epithelial allergy. C, Number of patients and samples used for each analysis. For further details, refer to Tables 1, Tables S1, and S2. II. Modulation of allergen-specific Ig by grass-tablet SLIT; Levels of sIgE (A.), sIgG4 (B.), and sIgE/sIgG4 ratio (C.) are shown as the $\log_2$ of x-fold change from baseline for the two main Phleum allergens, Phl p 1 + 5. 1st column graphs show data from all the patients in the study, 2s column graphs show data from Mono-sensitized patients and 3rd column graphs show data from Poly-sensitized patients. 4th column graphs show the trajectories for each patient. Continued lines represent the median and discontinued lines the quartiles in the Violin plots. *** $P \leq .001$, ** $P \leq .01$, * $P \leq .05$

might identify new biomarkers to allow future studies comparing different intervention strategies. It has been published that SLIT is also safe and effective in Poly-sensitized patients[15]; however, the SLIT management is not so clear as it is for Mono-sensitized patients who usually respond better to the treatment. This exploratory study has been designed in a double-blind placebo-controlled set-up. Untargeted metabolomics and transcriptomics as well as serological determinations comparing initial conditions and status at two years of treatment have been carried out, and the results analyzed in the light of the Mono- or Poly-sensitization status of patients. The most

TABLE 1 Patient Characteristics and Sensitization Profile

Subject Number	Treatment	Age	Sex	Ethnicity	*Phleum pratense* (CAP class)	Smoker	Asthma	Sensitiz. Profile
1	Active	31	F	Caucasian	3	No	No	Mono
2	Active	47	F	Caucasian	3	No	Yes	Poly-Epi
3	Placebo	35	M	Hispanic	4	No	Yes	Poly
4	Placebo	39	F	Caucasian	3	Previous	Yes	Poly
5	Active	51	F	Hispanic	4	No	Yes	Poly-Epi
6	Active	22	F	Caucasian	3	Yes	Yes	Poly-Epi
7	Placebo	33	M	Caucasian	4	No	No	Poly-Epi
8	Placebo	50	F	Hispanic	6	Previous	Yes	Poly
9	Placebo	28	F	Hispanic	4	No	No	Poly
10	Active	25	F	Caucasian	3	No	No	Poly-Epi
11	Active	37	F	Hispanic	4	No	Yes	Poly
12	Active	32	M	Caucasian	4	Yes	Yes	Poly
13	Active	25	M	Hispanic	4	No	No	Poly
14	Placebo	38	F	Caucasian	3	No	No	Poly
15	Active	30	F	Caucasian	2	Yes	No	Poly
16	Placebo	41	M	Hispanic	6	No	No	Mono
17	Active	59	M	Caucasian	3	Yes	No	Mono
18	Active	34	M	Hispanic	4	Yes	Yes	Poly-Epi
19	Placebo	19	F	Hispanic	5	No	Yes	Poly-Epi
20	Placebo	28	M	Caucasian	3	Previous	Yes	Poly
21	Placebo	21	F	Caucasian	3	Yes	Yes	Mono
22	Placebo	39	F	Hispanic	4	No	No	Mono
23	Placebo	36	F	Hispanic	3	No	Yes	Poly
24	Placebo	24	M	Caucasian	3	No	No	Poly
25	Placebo	47	F	Caucasian	2	No	Yes	Mono
26	Placebo	43	F	Caucasian	3	No	No	Mono
27	Active	53	M	Caucasian	2	No	No	Mono
28	Active	53	M	Hispanic	5	No	No	Mono
29	Active	38	M	Caucasian	3	Yes	Yes	Poly-Epi
30	Placebo	33	F	Caucasian	3	No	Yes	Mono
31	Placebo	34	M	Caucasian	2	No	Yes	Poly-Epi

Abbreviations: AE: Adverse effects; Cyp: *Cupressus*; D. f: *Dermatophagoides farinae*; D. pt: *D pteronyssinus*; F: Female; IMP: Investigational Medical Product; Lep: *Lepidoglyphus destructor*; LTP: Lipid Transfer Protein; M: Male; Mono: Mono-sensitized; OAS: Oral Allergy Syndrome; Ole: *Olea*; Phl: *Phleum*; Pla: *Platanus*; Plan: *Plantago*; Poly: Poly-sensitized; Poly-Epi: Poly-sensitized with epithelial allergy; Sal: *Salsola*.

SENSITIZATION (ISAC)					
	Poly-sensitized				
Unique seasonal: *Phleum pratense*	Multiple seasonal: Grass/Tree Pollen/Weed Pollen	Perennial: Epithelia	Perennial: Mites	Panalergen	AE with IMP[&]
Phl					Throat pruritus
Phl		Dog			Throat pruritus
Phl			D. pt	Profilin	Epigastric pain
	Phl, Cyp			Profilin	No
	Phl, Cyp, Ole	Dog	*Blomia*		Oral pruritus, SL and labial edema
	Phl, Cyp, Ole	Cat			Throat pruritus, dysphagia, dyspnea
	Phl, Cyp, Ole	Cat	Lep	Profilin	Oral pruritus
	Phl, Cyp, Ole, Pla, Sal			Profilin	No
	Phl, Cyp, Pla,				SL edema
	Phl, Cyp, Ole, Plan	Dog		Profilin	SL edema, dyspnea, foreign body sensation
Phl			D. pt		Dysphagia, chest tightness
	Phl, Cyp				No
	Phl, Cyp, Pla				SL edema, pharyngeal pruritus, lingual pruritus, oral pruritus
	Phl, Cyp				No
	Phl, Cyp, Ole				Oral pruritus
Phl				Polcalcin, Profilin	No
Phl					Oral pruritus, itchy tongue
	Phl, Cyp, Ole, Pla, Sal	Dog			Oral pruritus, abdominal pain
	Phl, Cyp, Pla,	Cat	D. f/pt		No
	Phl, Cyp, Ole, Pla				No
Phl					Pruritus
Phl					No
	Phl, Ole				OAS
	Phl, Cyp				No
Phl				LTP	No
Phl					Thirst
Phl					Oral and facial pruritus, foreign body sensation, pharyngeal edema, chest tightness
Phl					No
	Phl, Cyp, Ole, Pla,	Dog			Pharyngeal pruritus
Phl					No
	Phl, Cyp, Ole, Pla,	Dog			No

relevant systemic change detected was the desensitization of effector cells, which was only detected in Mono-sensitized patients after two years of SLIT, while other systemic inflammatory signals did not improve. These changes may be related to the clinical improvement, as previously reported.[1,2] The results of this study provide new ways of understanding the effect of SLIT and offer clues for future directions for SLIT intervention.

2 | MATERIALS AND METHODS

2.1 | Study design

Forty-seven adult patients were enrolled in this Phase IV exploratory randomized, parallel-group, double-blind, and placebo-controlled, 2-center national trial (EUDRA CT 2012-005092-14). Subjects were randomized (1:1). Twenty-three patients were assigned to daily sublingual administration of active (Grazax®). Detailed information is provided in Supporting Information (SI) Part 1.

The study consisted in 8 visits (V). In the first visit (V1), the screening and randomization of patients in two groups—Placebo and Active—was performed. At V2 (T0) the first blood sample was extracted, and the treatment started. Serum was extracted in visits 3 to 8 (V3-V8) during the corresponding treatment. Samples were obtained outside Grass Pollen Season (GPS) in visits V3, V4, V6, and V8, corresponding to 1 month, 4 months, 12 months, and 24 months after starting the treatment, respectively. Samples extracted in V5 and V7 occurred during two consecutive GPS. V8 will be referred to as T2 (two years of treatment). A schematic of the clinical trial is presented in Figures 1-I-A. Thirty-one subjects remained at T2, of which 14 were Active and 17 Placebo (Figure S1). Patients were also stratified according to their sensitization profile into Mono- or Poly-sensitized.

Inclusion/exclusion criteria followed approved indication for the product in the Summary of Product Characteristics. Patients with perennial rhinitis or perennial asthma were excluded. The institutional review board approved the study protocol; all subjects were informed of the aim of the trial and provided written consent.

2.2 | Sample collection and processing

For immunological analyses and metabolomics, serum samples were obtained and stored until analysis. In the case of transcriptomics, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque (GE Healthcare™, Chicago, Illinois, USA) density gradient centrifugation and stored in Buffer RLT until analysis.

2.3 | Allergen-specific IgE and IgG4

Allergen-specific IgE was measured using the ADVIA Centaur platform (Siemens Healthcare Diagnostics, Inc, Tarrytown, NY, United States) according to standard methods. Values provided are the sum of Phl p 1 and Phl p 5. Phl p 1- and p 5-specific IgG4 was determined by ELISA.[1] All samples were tested by ImmunoCAP-ISAC (Thermo Fisher Scientific, Uppsala, Sweden) to investigate sensitization profiles.

Statistical analysis was performed using IBM SPSS Statistics, v24. As data did not follow normality, Mann-Whitney U test was applied to test the differences in the treatment at each time point. On the other hand, Friedman test was used to obtain differences due to the time in each treatment. Moreover, Wilcoxon test was performed for pair-based comparisons. In all cases, P-values $< .05$ were considered significant. Data were represented using violin plots showing sample median ± max and min values with Prism v7.0 software (GraphPad Software, La Jolla, CA).

2.4 | Patient selection for Omics analysis

Patients with concomitant epithelial allergy (Poly-Epi) were excluded from all the omics analyses. Even if included patients sensitized to epithelia did not have the pet at home, they were excluded as the unnoticed exposure to the offending allergen is highly variable and may introduce a confounding factor.[16,17] Poly-sensitized patients were, in all cases but one, co-sensitized at least to grass and cypress. A detailed list with the number and the specific patients included in each omics analysis and each comparison is given in Table S1.

2.5 | Metabolomic analysis

Serum samples were measured using a multiplatform analysis: Liquid and Gas Chromatography coupled to Mass Spectrometry (LC-QTOF-MS Agilent series 6520 and GC-Q-MS Agilent series 5975C, respectively). Both techniques followed previously described methodologies developed in our group.[18,19]

Full descriptions are available in Supporting Information (SI-Part 2). Metabolite annotation was carried out using the online advanced CEU Mass Mediator tool.[20,21] These were confirmed through LC-MS/MS experiments using 20 eV for fragmentation. Data were uploaded to Metabolomics Workbench webpage (number: ST001352).

2.5.1 | Statistical analyses

Quality assurance (QA) of the data was tested, as previously described,[22,23] using principal component analysis (PCA) models in SIMCA (v.14.1, Umetrics®, Umeå, Sweden). Afterward, univariate analysis was performed in MATLAB (v.R2018b, MathWorks®, Natick, Massachusetts, USA) to obtain the p-value for each compound in the study. One-to-one comparisons using Mann-Whitney U test (MWU) with a Benjamini-Hochberg

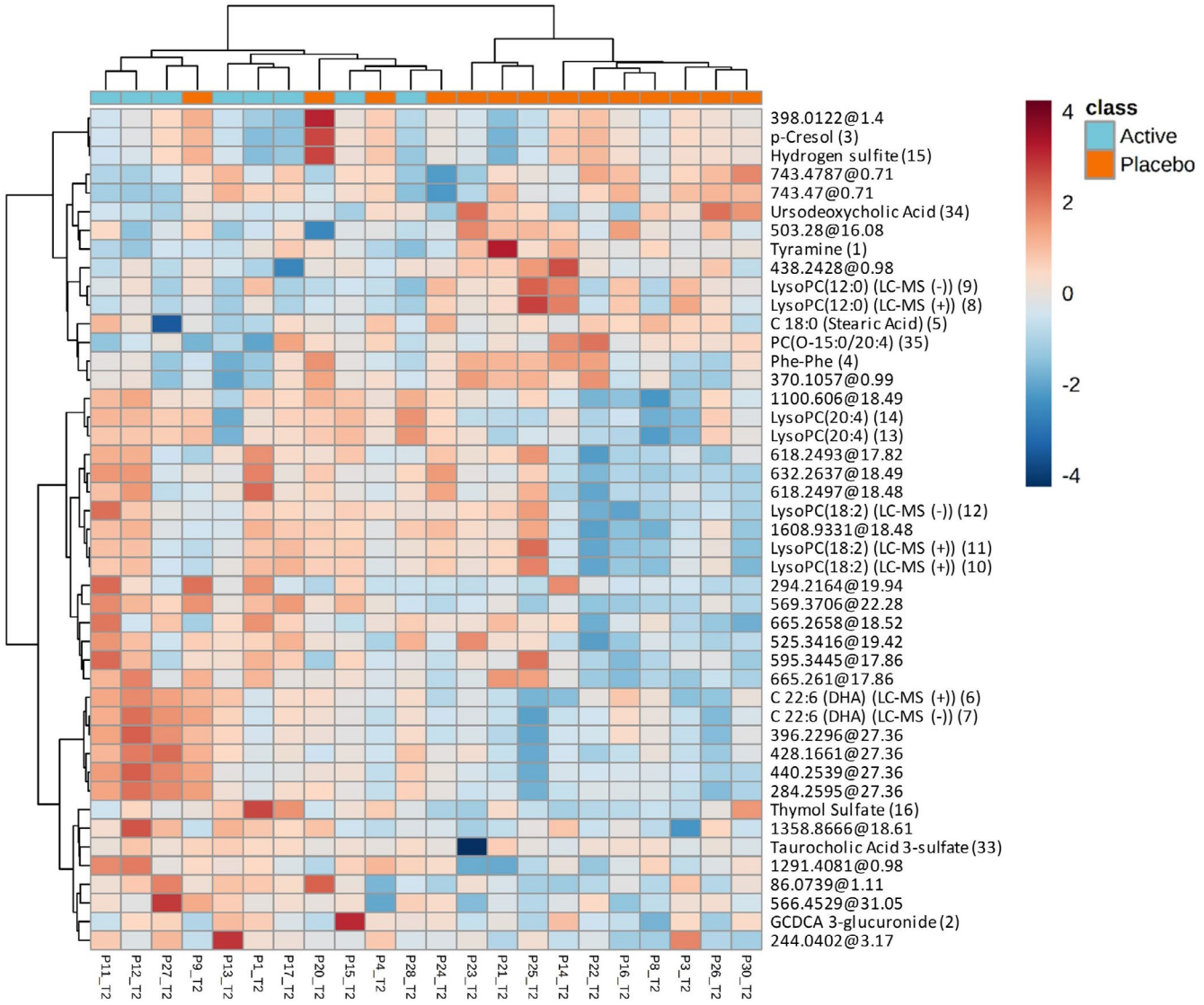


FIGURE 2 Significant signals from metabolomics between Active (n = 8) and Placebo (n = 14) groups at T2 were depicted on a heat map using hierarchical clustering of the samples (represented in columns) and metabolites (in rows). Red cells represent higher levels of the specific metabolite in that sample, whereas blue cells represent lower levels. Samples and metabolites are clustered according to their similarity. Mann-Whitney U test with a Benjamini-Hochberg correction was used to detect statistical significance ($P < .05$). Unknown features (metabolites without annotation) are represented by "Mass@Retention Time." Numbers in parentheses refer to the metabolite N° in Tables S5 and S6, where detailed information is available, including abbreviations

correction were performed, and statistical significance was set at 95% level ($P < .05$ for the adjusted p-value). The MetaboAnalyst online tool (v. 4.0) was used to produce heat maps with hierarchical clustering.[24] Euclidean distance measure and the Ward clustering algorithm were chosen as the clustering parameters.

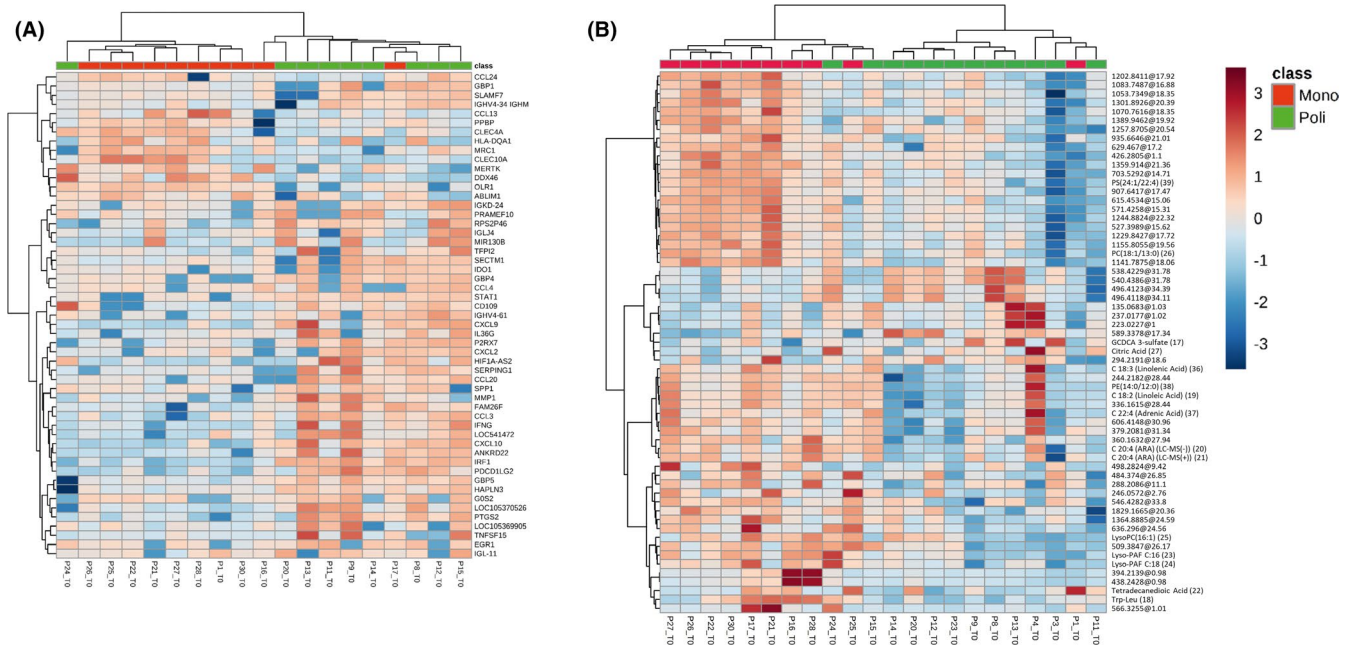
2.6 | Transcriptomic analysis

Isolated PBMCs were analyzed by transcriptomics using microarrays. Full descriptions of the methodologies used are available in Supporting Information (SI-Part 3).

2.6.1 | Statistical analysis of microarray data

Gene-level expression analysis was carried out with R 3.5.1 software using *limma* package. Those genes with a $\log_2$ fold change higher than 1 or lower than −1 and with a p-value less than 0.05 were considered as differentially expressed among the experimental groups.

The pathways and gene regulators involved were analyzed by Ingenuity Pathway Analysis (release from Dec 2019) (IPA, Qiagen) and R software. Gene Set Enrichment Analysis (GSEA) of the Robust Multi-array Average (RMA)-normalized samples was performed using gseKEGG function from *clusterprofiler* 3.8.1 R package with the following parameters: Organism database = org.Hs.eg.db, ontology = "ALL," number of permutations = 1000, adjusted p-value cutoff = 0.1. Raw data can



C

Poli vs Mono T0

Pathway	Analysis	*p-value*	Enrichment Score (ES)	Activation Z-Score	n
Inflammatory response	IPA	2.10E-14	-	2.349	21
Activation of blood cells	IPA	1.32E-11	-	2.959	18
Activation of leukocytes	IPA	2.64E-11	-	2.827	17
Migration of antigen presenting cells	IPA	5.77E-08	-	2.791	8
Toll-like receptor signaling pathway	GSEA	1.60E-03	0.899	-	5
IL-17 signaling pathway	GSEA	1.03E-02	0.818	-	5

FIGURE 3 Significant signals in transcriptomics (A.) and metabolomics (B.) between Mono (n = 10) and Poly (n = 9 in transcriptomics, 12 in metabolomics) groups at T0 were depicted on heat maps using hierarchical clustering of the samples (represented in columns) and transcripts/metabolites (in rows). Red cells represent higher levels of the specific transcript/metabolite in that sample, whereas blue cells represent lower levels. Samples and transcripts/metabolites are clustered according to their similarity. Mann-Whitney U test was used to detect statistical significance ($P < .05$). In B., unknown features (metabolites without annotation) are represented by "Mass@ Retention Time." Numbers in parentheses refer to the metabolite N° in Tables S5 and S6, where detailed information is available, including abbreviations. C. IPA and GSEA significant transcriptomics results for "Mono *vs* Poly" comparison at T0

be found on GEO database with the accession number GSE147197. Heat maps with hierarchical clustering were produced as for metabolomics.

3 | RESULTS

3.1 | Patient allergic phenotype and characteristics

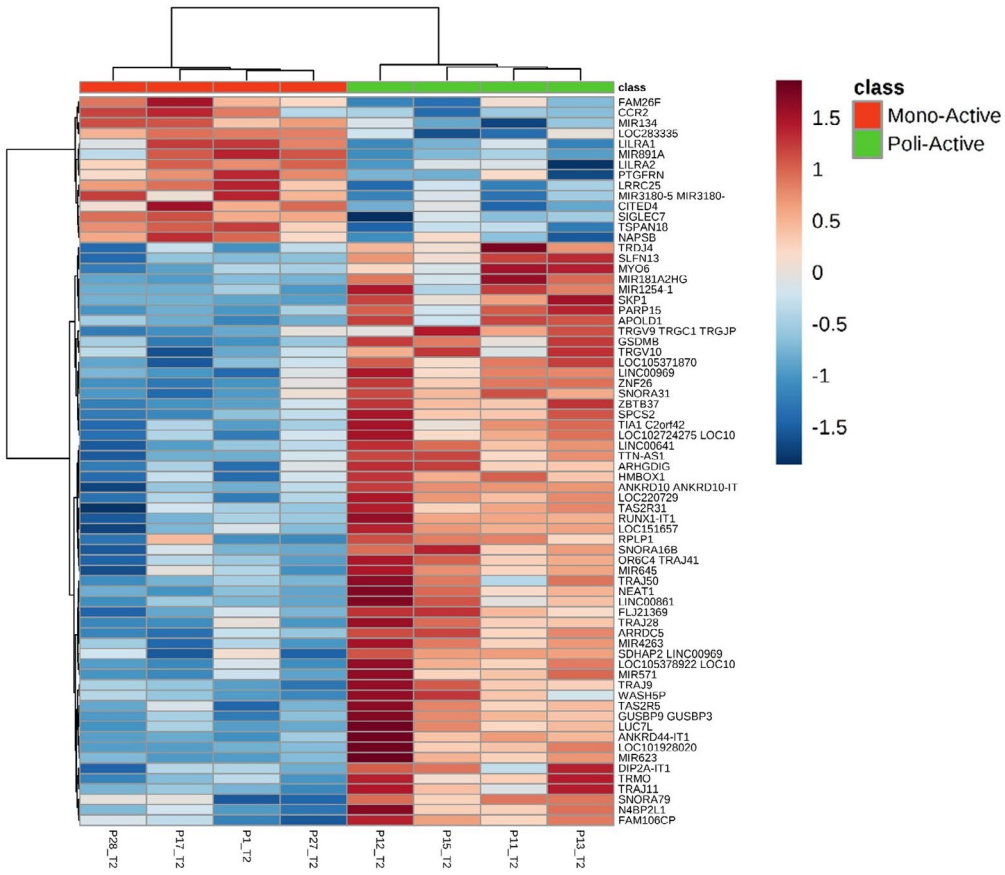
The study was completed by 31 (70%) of the enrolled patients, 17 Placebo and 14 Active (clinical characteristics in Table 1), mean age of 36 (±10.3) years old and sex-matched (58% female). Additionally, *Phleum pratense* CAP class was mostly 3 and 4 (74%). 22% and 10% of the patients were active or previous smokers, respectively, and 53% had asthma. Furthermore, 32% of the patients were Mono-sensitized (Mono) to *Phleum*, whereas those Poly-sensitized (Poly) were sensitized either to seasonal or perennial allergens. Detailed information is provided in Table 1 and Figures 1-I-B. In addition, clinical data according to treatment and to the patient sensitization profile were summarized in Table S2. The groups were balanced in terms of sex, smoking habits, ethnicity, asthma, and sensitization status, and no statistical difference was observed for any of the clinical data between Active/Placebo or Mono/Poly groups. A detailed description of the patients included in each analysis is provided in Figures 1-I-C and Table S1.

3.2 | sIgE and sIgG4 kinetics for Active SLIT and Placebo

In accordance with previous publications,[2] after 1 month of SLIT, an increment in the production of sIgE was observed in the Active group with a progressive decline during the two years of treatment. Mono and Poly groups showed the same trend, although the Mono group showed higher sIgE than Poly, especially from 4 months to the end of the treatment (Figures 1-II-A).

On the other hand, sIgG4 levels presented up to a 40-fold increment from their baseline after 12 months of the treatment in the

(A)



(B)

Poli-Active vs Mono-Active T2					
Pathway	**Source**	***p-value***	**Enrichment Score (ES)**	**Activation z-score**	**n**
Infiltration by T lymphocytes	IPA	3,57E-04	--	-2,019	7
Lymphocyte migration	IPA	6,40E-04	--	-2,035	13
Recruitment of leukocytes	IPA	3,11E-06	--	-2,109	16
T cell migration	IPA	1,23E-04	--	-2,118	12
Cell movement of leukocytes	IPA	2,80E-04	--	-2,162	24
Chemotaxis of mononuclear leukocytes	IPA	1,07E-03	--	-2,224	8
Polarization of blood cells	IPA	2,31E-03	--	-2,387	6
Calcium signaling pathway	GSEA	4,6E-03	-0,937	--	6

FIGURE 4 A. Significant signals from transcriptomics between Mono-Active (n = 4) and Poly-Active (n = 4) at T2 were depicted on a heat map using hierarchical clustering of the samples (represented in columns) and transcripts (in rows). Red cells represent higher levels of the specific transcript in that sample, whereas blue cells represent lower levels. Samples and transcripts are clustered according to their similarity. Mann-Whitney *U* test was used to detect statistical significance ($P < .05$). B. IPA and GSEA significant results for "Mono-Active vs Poly-Active at T2" comparison

Active group that was sustained until the end of the study (Figures 1-II-B). This increment was higher in Poly than in Mono. These trajectories of sIgE and sIgG4 were not observed in the Placebo group.

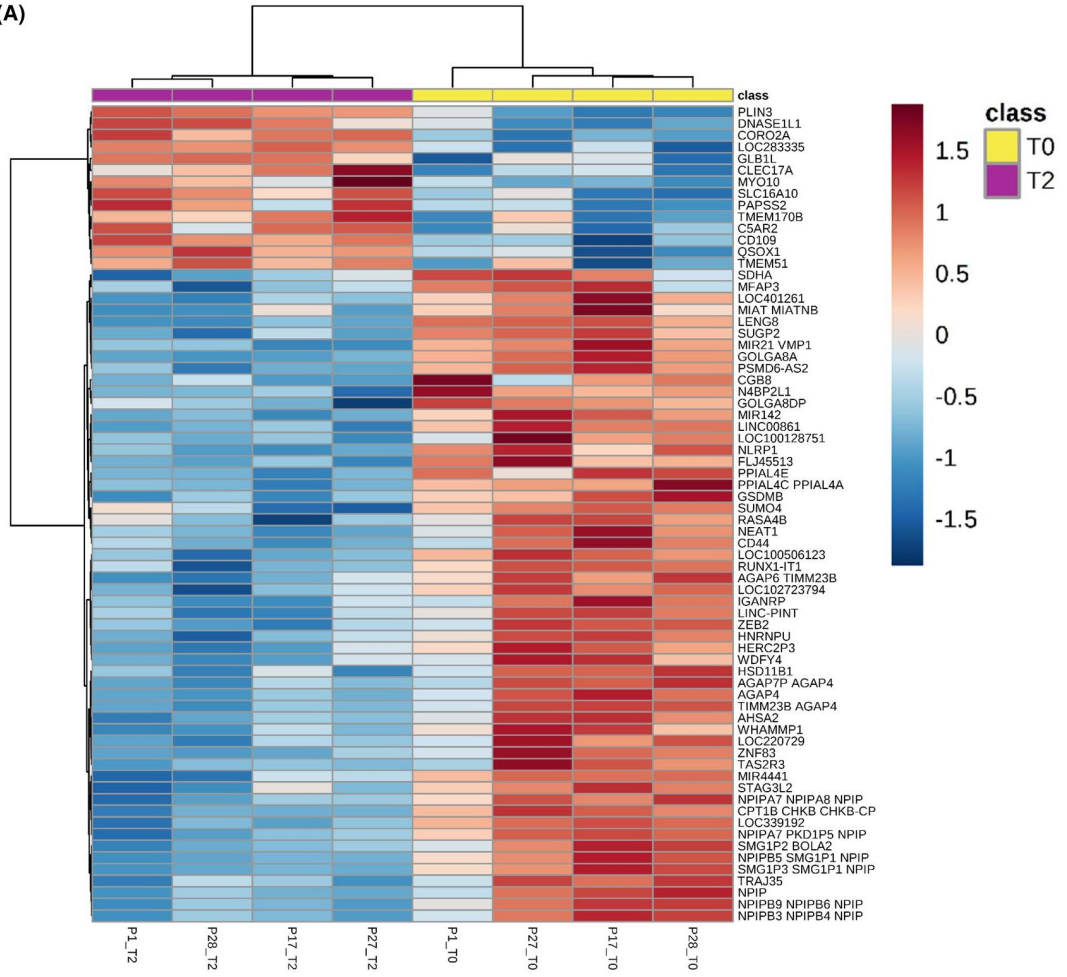
Additionally, sIgE/sIgG4 ratio was calculated. This was significantly higher after 1 month of treatment in the Active group, but from GPSI, the ratios fall below Placebo values (Figures 1-II-C). Statistical differences among time for Active and Placebo can be found in Table S3. Finally, patient trajectory plots showed that the same person had always higher values of sIgE, sIgG4, and their ratio over time (Figures 1-II, 4th column). Raw data of sIgE and sIgG4 can be found in Table S4.

To sum up, Active and Placebo groups showed the previously described behavior during SLIT.

3.3 | Active SLIT and Placebo patients display a differential metabolomic fingerprint at 2 years of treatment

Metabolomic and transcriptomic profiles of all the subjects included in the study were obtained. The metabolomic profiles consisted of 1259 and 67 entities from LC-MS (positive and negative ionization

(A)



(B)

Mono-Active T2 vs Mono-Active T0					
Pathway	**Analysis**	***p-value***	**Enrichment Score (ES)**	**Activation Z-Score**	**n**
Function of blood cells	IPA	2.91E-05	-	-	10
Degranulation of mast cells	IPA	8.74E-04	-	-2.000	6
Degranulation of phagocytes	IPA	1.39E-03	-	-2.000	12
Glucose metabolism disorder	IPA	3.17E-03	-	-1.982	25
Purine metabolism	GSEA	4.29E-03	0.957	-	2
Metabolic pathways	GSEA	5.80E-03	0.536	-	15
Arachidonic acid metabolism	GSEA	4.93E-02	0.872	-	2

FIGURE 5 A. Significant signals from transcriptomics between Mono-Active T2 (n = 4) and Mono-Active T0 (n = 4) were depicted on a heat map using hierarchical clustering of the samples (represented in columns) and transcripts (in rows). Red cells represent higher levels of the specific transcript in that sample, whereas blue cells represent lower levels. Samples and transcripts are clustered according to their similarity. Mann-Whitney U test was used to detect statistical significance ($P < .05$). B. IPA and GSEA significant results for "Mono-Active T2 vs Mono-Active T0" comparison

modes together) and GC-MS, respectively. These entities passed the QA of each technique by the clustering of quality control samples in the PCA model as can be seen in Figure S2. A complete transcriptomic analysis using GeneChip Human Gene 2.1 ST strips was also performed, where 48.000 transcripts were analyzed.

First, we set out to analyze whether there were differences between Active and Placebo groups at T2. Using metabolomics, we found 45 significant features, which were represented using a heat map with a hierarchical clustering (Figure 2). After their annotation, 19 metabolites were obtained encompassing lysophospholipids (*eg*, LysoPC 20:4, LysoPC 18:2), bile acid conjugates, and fatty acids (*eg*, stearic acid and docosahexaenoic acid, DHA) (Table S5A). These metabolites were not significant at the basal level (T0). Detailed information about the physicochemical properties such as formula,

biochemical class, and analytical parameters of the significant metabolites is presented in Table S6. These results suggest differences in the metabolomic profile of Active and Placebo patients at 2 years of treatment. No significant differences were found in transcriptomics.

3.4 | Differences in the patient sensitization profile at T0 lead to differential omics profiles

As opposed to Mono patients, Poly patients suffer allergenic exposure in different months of the year; thus, we presumed that this would influence their systemic inflammatory-associated signatures, which would differ from those of Mono patients.

When compared at T0, we found 53 significant signals in transcriptomics that are represented in Figure 3A. These signals were able to group correctly 85% of the samples. In metabolomics, 61 significant signals were found and are presented in Figure 3B. Moreover, we performed pathway analysis of significant transcripts using IPA and GSEA, aiming to match these transcriptomic differences among the two phenotypes with biological processes (Figure 3C). GSEA and IPA significant results indicated that pathways related to inflammatory processes such as activation of leukocytes and blood cells, inflammatory response, TLR, and IL17 were positively correlated (positive Enrichment Score in GSEA) or activated (positive Activation Z-Score in IPA) in the Poly subjects. These results suggest that Poly subjects present a higher degree of inflammation than Mono subjects. Complementary to these results, in metabolomics, 15 out of 61 significant entities were annotated, including fatty acids (eg, linoleic acid and arachidonic acid (AA)), phospho- and lysophospholipids (eg, Lyso-PAF C:16, Lyso-PAF C:18, LysoPC(16:1)) and small organic molecules such as citric acid (Table S5B).

To sum up, Poly and Mono patients present different metabolic and transcriptomic profiles prior to SLIT treatment. These signatures point to a higher inflammatory profile in Poly-sensitized patients.

3.5 | The importance of the sensitization profile of Active patients at two years of treatment

To assess the differences between Mono and Poly patients after two years of treatment, Mono-Active and Poly-Active patients were compared at T2. The significant transcripts were represented in Figure 4A. All patients were correctly grouped, and a very distinct signature could be appreciated between them. Regarding the pathway analysis, IPA results showed that routes related to infiltration and migration of T lymphocytes and leukocytes as well as blood polarization pathway had a negative Z-score, meaning that they were inactivated in Poly-Active patients. Moreover, calcium signaling pathway was negatively correlated with Poly-Active patients in GSEA analysis (Figure 4B).

Regarding metabolomics, 6 significant entities out of 16 were annotated, encompassing a bile acid (deoxycholic acid 3-glucuronide), an amino acid derivative (hydroxy-proline), and sugar-derived metabolites (eg, erythritol). They are presented in Table S5C.

These results suggest that Mono-Active and Poly-Active patients are different at two years of treatment, and that Mono-Active patients show higher levels of inflammation at this point.

3.6 | Mono patients after 2 years of SLIT treatment display a transcriptomic signature associated with effector cell downregulation

As Mono patients are known to respond better to SLIT, we set out to find the specific differences caused by two years of SLIT in this group. Thus, the transcriptomic profiles of the Mono-Active patients between T0 and T2 were compared. In the resulting heat map (Figure 5A), the hierarchical clustering of the samples displayed a total separation between T0 and T2. Interestingly, most of the significant transcripts in the heat map are downregulated at T2. Additionally, pathway analysis indicated that glucose and AA metabolism were positively correlated with T2, and pathways such as mast cell and phagocyte degranulation were inactivated at T2 but not at the beginning of the treatment (Figure 5B).

These results suggest that SLIT has a significant effect on essential cellular mechanisms taking place along SLIT response in Mono-Active patients.

4 | DISCUSSION

Understanding systemic effects induced by SLIT is essential for multiple reasons. First, for the proper positioning of SLIT within the pharmacological portfolio in allergy. Second, to select adequate SLIT candidates and to build an alliance between prescriber and patient that would promote compliance and intervention success. Last, to identify new and relevant biomarker strategies that would be used to select SLIT candidates, to monitor effects, and to prove the unique value of SLIT as a disease-modifying intervention.

Omics methodologies used in combination provide very potent tools to analyze systemic signatures that have proven recently their value for understanding allergic disease progression.[13] Thus, we decided to use a similar approach in a prospective, two-year, double-blinded placebo-controlled SLIT trial. We used Grazax® as it is the SLIT product with the best documentation and the only one with three five-years prospective clinical trials.[7] Moreover, we know that when administered during three consecutive years, it induces sustained benefit at least two years after discontinuation,[2] but if administered only two, the effect is quickly lost.[6] This loss is equal for vaccines administered by subcutaneous route, and an adequate explanation for it is missing.[25]

In a recently published five-years prospective study with the same SLIT product, Varona et al demonstrated the existence of multiple mechanisms that involved effector cell desensitization (acting early) and T-cell regulation after the second year of treatment.[2] The

precise sequence of these mechanisms was not clear. T regulation seems to be established only in the second year of intervention, while meaningful systemic signatures such as sIgE and eosinophils counts only improved in the third year. This last result agrees with GAP clinical trial data on bronchial hyperreactivity.[9]

All the previous data point to the second year of intervention as the critical crossroad of effect mechanisms and thus the best time point to perform the first exploratory trial on systemic effects induced by SLIT.

In this sense, we compared Active and Placebo groups, as well as Mono- and Poly-sensitized patients. Finally, we compared Mono-sensitized patients before and after intervention.

4.1 | Active vs Placebo

Interesting differences between Active and Placebo groups at T2 were found. We detected four main observations: First, an increase of lysophosphatidylcholine 20:4 (LysoPC(20:4)) and LysoPC(18:2), which are molecules that could release arachidonic acid (AA, 20:4) and linoleic acid (18:2), respectively, by the action of the Phospholipase A2.[26] This enzyme is activated upon different stimuli, including inflammation.[27] These changes can be associated with a stimulation of the AA pathway, pointing to an inflammatory response due to the stimulation by the Active medication, although AA metabolism is complex and could also lead to anti-inflammatory mediators.[28] Second, we observed an increase of Docosahexaenoic Acid (DHA) in the Active group. This metabolite is also from AA metabolism and has been associated with the inhibition of leukocyte chemotaxis and suppression of pro-inflammatory cytokines and eicosanoids.[29] As a consequence, its increment can be associated with a regulation pathway to cope with inflammation. Third, tyrosine catabolism can produce tyramine and phenols such as p-cresol by the gut microbiome.[30] Regarding p-cresol, this metabolite has shown to both decrease the integrity of the gut epithelium and the viability of intestinal epithelial cells.[31] The decrease of these two metabolites in the Active group might reflect one of the positive effects of the treatment. Finally, we observed an increase of hydrophilic bile acid conjugates (Taurocholic acid 3-sulfate and Glycochenodeoxycholic Acid 3-glucuronide) in the Active group. These molecules are associated with anti-inflammatory properties.[32] None of these metabolites were significantly different at T0, so these differences are most probably caused by the treatment.

4.2 | Mono- versus Poly-sensitized patients

In addition, we wanted to test the differences between the patients regarding the sensitization profile before the treatment. Thus, we delved into the comparison between Poly and Mono at T0. When observing metabolomics, the results showed lower levels of linoleic acid, linolenic acid (18:3), AA, and adrenic acid (22:4) in Poly patients, suggesting that the pathway of synthesis of AA is altered.[26] We hypothesize that these fatty acids are being used to synthesize inflammatory mediators due to a higher inflammatory state in the Poly patients. Furthermore, lower levels of Lyso-PAF C:16 and Lyso-PAF C:18 were observed. The former is a precursor of the platelet-activating factor (PAF), an important inflammatory mediator.[33] These lower levels in its precursors could signify enhanced synthesis of PAF. This is supported by transcriptomics results, where activation of blood cells and IL-17 pathways are activated in Poly patients. Moreover, we observed higher levels of citric acid, a compound that has shown antioxidant and anti-inflammatory properties.[34] This may suggest the activation of antioxidant pathways to make up for the enhanced oxidative stress caused by allergic inflammation. In addition, other transcriptomic pathways related to inflammatory response (such as antigen- presenting cells, activation of leukocytes, and Toll-like receptor signaling pathway) are also activated in Poly patients. To sum up, all these results may support the idea that Poly patients have higher inflammatory state.

Moreover, the differences between Mono-Active and Poly-Active at T2 were observed and were most evident by transcriptomics, with a clear signature between these groups. Pathways such as infiltration and migration of T lymphocytes and leukocytes as well as blood polarization pathway were inactivated in Poly-Active patients, in addition to calcium signaling pathway. These results suggest that Mono-Active patients present higher levels of inflammation at T2.

4.3 | Evolution of Mono-sensitized patients during SLIT

Mono-Active patients were also analyzed over time to evaluate the differences due to the SLIT treatment. In this case, pathway analysis showed that effector cells, such as mast cells and phagocytes, present lower levels of activity at T2. This suggests that the SLIT is capable of inducing desensitization and that it is maintained during all the active treatment phase. In addition, transcripts from the metabolism of AA were upregulated at T2, which supports the idea of a higher level of inflammation at T2.

In summary, while there is not any systemic inflammatory signature improvement, there is a strong downregulation of effector cell functionality. This effect, interestingly, is detected in PBMCs, a fact that suggests an interconnection of peripheral and local effector cell networks.

This effect is not observed in Poly-sensitized subjects (data not shown). This is aligned with the clinical observation that desensitization in SLIT is allergen-specific.[35]

4.4 | Implications for SLIT future

The results of our study clearly suggest that the administration of SLIT for two years is associated to effector cell desensitization. Peripheral improvement is not detected, which might explain why discontinuation during this phase causes an immediate loss of

therapeutic benefit. This fact is of pivotal importance for the correct administration of SLIT and would explain many of the failed trials with a focus on T regulation performed only for 1-2 years, as has been previously discussed in Varona et al[2]

Further investigation is needed to understand underlying mechanisms associated to this desensitization. In particular, (a) why it is antigen-specific, (b) how this desensitizing process is transferred from a direct desensitization signal in the site of administration to a systemic effect, and (c) how effector cell control is achieved by regulatory networks in the third year of intervention.

As effector cells produce multiple inflammatory mediators that contribute to sustain Th2 including prostaglandins, leukotrienes, cytokines, and inflammatory mediators, early desensitization might be essential for a later regulatory response.[7,36-38] Unfortunately, we lack long-term SLIT studies with products tailored only for T-cell recognition, so we cannot answer this question, which would be essential for the design of successful new SLIT intervention strategies.

A second objective of the study was to evaluate new potential biomarkers for monitoring SLIT effect. This objective might be difficult to achieve. First of all, there is no clear peripheral benefit during the first two years of intervention. Second, there is high individual variability. One of the most relevant confounding factors is the co-existence of other sensitizations. For example, a variation in a second sensitization exposure, as is the case of cypress pollen in our study, has the potential of influencing overall inflammatory phenotype of the study group. In our case, the inclusion phase—which followed product indications—overlapped with cypress pollen season, while clinical screening for effects did not.

Perhaps we should change the scope. In a recent publication, Obeso et al described unique metabolomic and transcriptomic systemic signatures associated to severe grass-allergic phenotypes.[13] These signatures pointed to altered energy metabolism, systemic uncontrolled inflammation, and collapse of repair systems. These biomarkers could be used to identify and exclude such patients for SLIT intervention. It is clear from the recent studies that the first phase of SLIT is associated to an increase in peripheral inflammatory phenotype,[1,2] that is progressively improving thereafter. From Obeso et al results, it is clear that some patients might not be able to deal with this increase. We are currently in the process of validation of this new biomarker approach, which could be common for different intervention strategies.

Additionally, in this work we present complimentary sets of omics data; however, further integration analysis should be performed in order to generate a complete and comprehensive analysis of the underlying mechanisms of SLIT. Lastly, future prospective studies in bigger cohorts will allow for a deeper analysis of the metabolomic and transcriptomic signatures and clarify their link to a clinical effect.

From the accumulated evidence with Grazax studies, it becomes apparent that a systemic benefit should be achieved after 3 years of intervention and two additional years of patient follow-up. We could use this new biomarker strategy to prove the value of SLIT intervention. Obviously, this approach should not be of value for individual patient management but might be essential for the positioning of SLIT and etiological allergy management as the central therapeutic approach in allergic diseases.

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AUTHOR CONTRIBUTIONS

DB, TC, C.Blanco, and MFR were the PI. RB, S.VC., TR, C. Blanco, and MFR included all study patients and the clinical data. PR coordinated the clinical trial. C. Barbas and AV, and MME supervised the metabolomic and transcriptomic analyses, respectively. L.M-B. and DR performed the transcriptomic analysis and data treatment. DO, RB, AV, and TCB-T. performed the metabolomics analysis and data treatment. All authors contributed to the writing of the manuscript and have given approval to the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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