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

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Abbreviations:

AA: Arachidonic acid

AIT: Allergen immunotherapy

FC: Fold change

GC-MS: Gas chromatography coupled to mass spectrometry

GSEA: Gene Set Enrichment Analysis

IPA: Ingenuity Pathway Analysis

LC-MS: Liquid chromatography coupled to mass spectrometry

LPCs: Lysophosphocholines

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

IMP: Investigational Medical Product

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 Poli-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 Poli-sensitized groups, excluding patients allergic to epithelia. Individuals were compared based on their treatment (Active/Placebo) and sensitization level (Mono/Poli).

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. Poli-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 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.

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

AIT 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 analysed 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: (i) the need of administering the therapy daily for three consecutive years; (ii) the lack of adequate predictive biomarkers, and (iii) 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, might identify new biomarkers to allow future studies comparing different intervention strategies. It has been published that SLIT is also safe and effective in Poli-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 analysed in the light of the Mono or Poli-sensitization status of patients. The most 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.

MATERIALS AND METHODS

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 **Supplementary 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 **Figure 1-I-A**. Thirty-one subjects remained at T2, of which 14 were Active and 17 Placebo (**Figure S-1**). Patients were also stratified according to their sensitization profile into Mono- or Poli-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.

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.

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 (ThermoFisher 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 <0.05 were considered significant. Data were represented using violin plots showing sample median $\pm$ max and min values with Prism v7.0 software (GraphPad Software, La Jolla, CA).

Patient selection for Omics analysis

Patients with concomitant epithelial allergy (Poli-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} Poli-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 S-1**.

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 supplementary 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 was uploaded to Metabolomics Workbench webpage (number: ST001352).

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). Afterwards, 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 correction were performed, and statistical significance was set at 95% level ($p < 0.05$ for the adjusted p -value). The MetaboAnalyst online tool (v. 4.0) was used to produce heatmaps with hierarchical clustering.²⁴ Euclidean distance measure and the Ward clustering algorithm were chosen as the clustering parameters.

Transcriptomic analysis

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

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

RESULTS

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 ($\pm$ 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 Poli-sensitized (Poli) were sensitized either to seasonal or perennial allergens. Detailed information is provided in **Table 1** and **Figure 1-I-B**. In addition, clinical data according to treatment and to the patient sensitization profile were summarized in **Table S-2**. 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/Poli groups. A detailed description of the patients included in each analysis is provided in **Figure 1-I-C** and **Table S-1**.

slgE and slgG4 kinetics for Active SLIT and Placebo

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

On the other hand, slgG4 levels presented up to a 40-fold increment from their baseline after 12 months of the treatment in the Active group that was sustained until the end of the study (**Figure 1-II-B**). This increment was higher in Poli than in Mono. These trajectories of slgE and slgG4 were not observed in the Placebo group.

Additionally, slgE/slgG4 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 (**Figure 1-II-C**). Statistical differences among time for Active and Placebo can be found in **Table S-3**. Finally, patient trajectory plots showed that the same person had always higher values of slgE, slgG4 and their ratio over time (**Figure 1-II, 4th column**). Raw data of slgE and slgG4 can be found in **Table S-4**.

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

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 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 S-2**. 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 if there were differences between Active and Placebo groups at T2. Using metabolomics, we found 45 significant features, which were represented using a heatmap with a hierarchical clustering (**Figure 2**). After their annotation, 19 metabolites were obtained encompassing lysophospholipids (*e.g.* LysoPC 20:4, LysoPC 18:2), bile acid conjugates and fatty acids (*e.g.* stearic acid and docosahexaenoic acid, DHA) (**Table S-5A**). 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 S-6**. 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.

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

As opposed to Mono patients, Poli 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 Poli subjects. These results suggest that Poli 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 (e.g. linoleic acid and arachidonic acid (AA)), phospho- and lysophospholipids (e.g. Lyso-PAF C:16, Lyso-PAF C:18, LysoPC(16:1)) and small organic molecules such as citric acid (**Table S-5B**).

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

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

To assess the differences between Mono and Poli patients after two years of treatment, Mono-Active and Poli-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 Poli-Active patients. Moreover, calcium signaling pathway was negatively correlated with Poli-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 (e.g. erythritol). They are presented in **Table S-5C**.

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

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 heatmap (**Figure 5A**), the hierarchical clustering of the samples displayed a total separation between T0 and T2. Interestingly, most of the significant transcripts in the heatmap 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.

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 Poli-sensitized patients. Finally, we compared Mono-sensitized patients before and after intervention.

Active vs Placebo

Interesting differences between Active and Placebo groups at T2 were found. We detected four main observations: First, an increase of lysophosphocholine 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 leucocyte 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.

Mono- versus Poli-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 Poli 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 Poli 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 Poli 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 Poli 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 leucocytes and Toll-like receptor signaling pathway) are also activated in Poli patients. To sum up, all these results may support the idea that Poli patients have higher inflammatory state.

Moreover, the differences between Mono-Active and Poli-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 Poli-Active patients, in addition to calcium signaling pathway. These results suggest that Mono-Active patients present higher levels of inflammation at T2.

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 down-regulation 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 Poli-sensitized subjects (data not shown). This is aligned with the clinical observation that desensitization in SLIT is allergen-specific.³⁵

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, (i) why it is antigen-specific, (ii) how this desensitizing process is transferred from a direct

desensitization signal in the site of administration to a systemic effect, and (iii) 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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Figure Legends

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; Poli: Poli-sensitized; Poli-Epi: Poli-sensitized with epithelial allergy. **C).** Number of patients and samples used for each analysis. For further details refer to **Tables 1, S-1 and S-2.**

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, **2nd column** graphs show data from Mono-sensitized patients and **3rd column** graphs show data from Poli-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. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Figure 2. Significant signals from metabolomics between Active (n= 8) and Placebo (n=14) groups at T2 were depicted on a heatmap 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 ($p < 0.05$). Unknown features (metabolites without annotation) are represented by "Mass@Retention Time". Numbers in parentheses refer to the metabolite N° in **Tables S-5 and S-6**, where detailed information is available, including abbreviations.

Figure 3. Significant signals in transcriptomics (**A.**) and metabolomics (**B.**) between Mono (n=10) and Poli (n=9 in transcriptomics, 12 in metabolomics) groups at T0 were depicted on heatmaps 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 ($p < 0.05$). In **B.**, unknown features (metabolites without annotation) are represented by “Mass@Retention Time”. Numbers in parentheses refer to the metabolite N° in **Tables S-5** and **S-6**, where detailed information is available, including abbreviations. **C.** IPA and GSEA significant transcriptomics results for “Mono vs Poli” comparison at T0.

Figure 4. A. Significant signals from transcriptomics between Mono-Active (n=4) and Poli-Active (n=4) at T2 were depicted on a heatmap 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 ($p < 0.05$). **B.** IPA and GSEA significant results for “Mono-Active vs Poli-Active at T2” comparison.

Figure 5. A. Significant signals from transcriptomics between Mono-Active T2 (n=4) and Mono-Active T0 (n=4) were depicted on a heatmap 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 ($p < 0.05$). **B.** IPA and GSEA significant results for “Mono-Active T2 vs Mono-Active T0” comparison.

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Table 1. Patient Characteristics and Sensitization Profile

Subject Number	Treatment	Age	Sex	Ethnicity	Phleum pratense (CAP class)	Smoker	Asthma	Sensitiz. Profile	SENSITIZATION (ISAC)					AE with IMP®
									Unique seasonal: Phleum pratense	Polisensitized			Panalergen	
										Multiple seasonal: Grass/Tree Pollen/Weed Pollen	Perennial			
											Epithelia	Mites		
1	Active	31	F	Caucasian	3	No	No	Mono	Phl					Throat pruritus
2	Active	47	F	Caucasian	3	No	Yes	Poli-Epi	Phl		Dog			Throat pruritus
3	Placebo	35	M	Hispanic	4	No	Yes	Poli	Phl			D. pt	Profilin	Epigastric pain
4	Placebo	39	F	Caucasian	3	Previous	Yes	Poli		Phl, Cyp			Profilin	No
5	Active	51	F	Hispanic	4	No	Yes	Poli-Epi		Phl, Cyp, Ole	Dog	Blomia		Oral pruritus, SL and labial oedema
6	Active	22	F	Caucasian	3	Yes	Yes	Poli-Epi		Phl, Cyp, Ole	Cat			Throat pruritus, dysphagia, dyspnea
7	Placebo	33	M	Caucasian	4	No	No	Poli-Epi		Phl, Cyp, Ole	Cat	Lep	Profilin	Oral pruritus
8	Placebo	50	F	Hispanic	6	Previous	Yes	Poli		Phl, Cyp, Ole, Pla, Sal			Profilin	No
9	Placebo	28	F	Hispanic	4	No	No	Poli		Phl, Cyp, Pla,				SL oedema
10	Active	25	F	Caucasian	3	No	No	Poli-Epi		Phl, Cyp, Ole, Plan	Dog		Profilin	SL oedema, dyspnea, foreign body sensation
11	Active	37	F	Hispanic	4	No	Yes	Poli	Phl			D. pt		Dysphagia, chest tightness
12	Active	32	M	Caucasian	4	Yes	Yes	Poli		Phl, Cyp				No
13	Active	25	M	Hispanic	4	No	No	Poli		Phl, Cyp, Pla				SL oedema, pharyngeal pruritus, lingual pruritus, oral pruritus
14	Placebo	38	F	Caucasian	3	No	No	Poli		Phl, Cyp				No
15	Active	30	F	Caucasian	2	Yes	No	Poli		Phl, Cyp, Ole				Oral pruritus
16	Placebo	41	M	Hispanic	6	No	No	Mono	Phl				Polcalcin, Profilin	No
17	Active	59	M	Caucasian	3	Yes	No	Mono	Phl					Oral pruritus, itchy tongue
18	Active	34	M	Hispanic	4	Yes	Yes	Poli-Epi		Phl, Cyp, Ole, Pla, Sal	Dog			Oral pruritus, abdominal pain
19	Placebo	19	F	Hispanic	5	No	Yes	Poli-Epi		Phl, Cyp, Pla,	Cat	D. f/pt		No
20	Placebo	28	M	Caucasian	3	Previous	Yes	Poli		Phl, Cyp, Ole, Pla				No
21	Placebo	21	F	Caucasian	3	Yes	Yes	Mono	Phl					Pruritus
22	Placebo	39	F	Hispanic	4	No	No	Mono	Phl					No

23	Placebo	36	F	Hispanic	3	No	Yes	Poli		Phl, Ole				OAS
24	Placebo	24	M	Caucasian	3	No	No	Poli		Phl, Cyp				No
25	Placebo	47	F	Caucasian	2	No	Yes	Mono	Phl				LTP	No
26	Placebo	43	F	Caucasian	3	No	No	Mono	Phl					Thirst
27	Active	53	M	Caucasian	2	No	No	Mono	Phl					Oral and facial pruritus, foreign body sensation, pharyngeal oedema, chest tightness
28	Active	53	M	Hispanic	5	No	No	Mono	Phl					No
29	Active	38	M	Caucasian	3	Yes	Yes	Poli-Epi		Phl, Cyp, Ole, Pla,	Dog			Pharyngeal pruritus
30	Placebo	33	F	Caucasian	3	No	Yes	Mono	Phl					No
31	Placebo	34	M	Caucasian	2	No	Yes	Poli-Epi		Phl, Cyp, Ole, Pla,	Dog			No

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

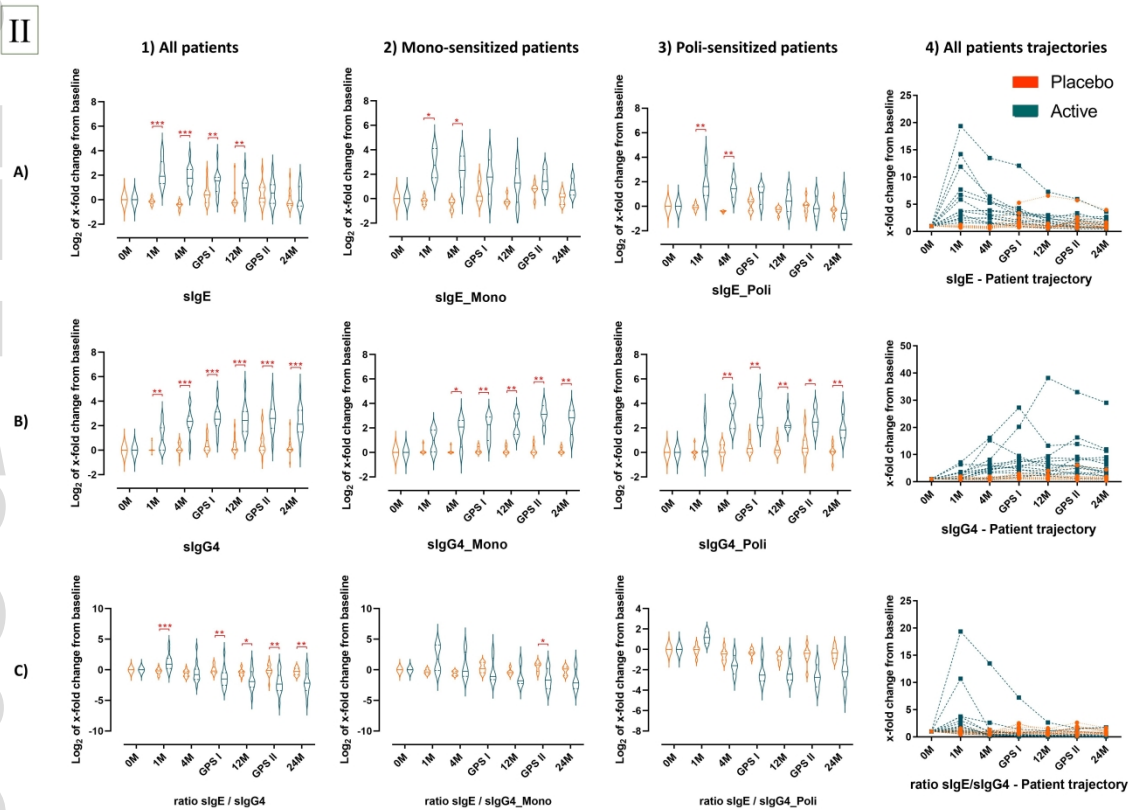
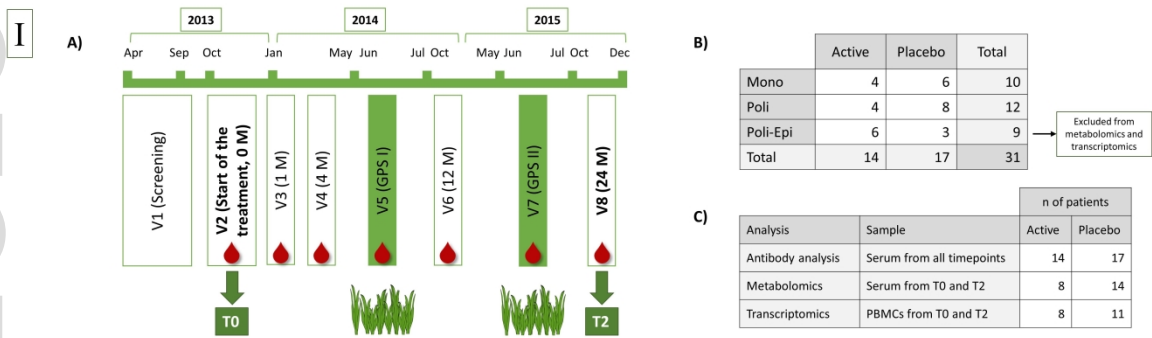


Figure 1

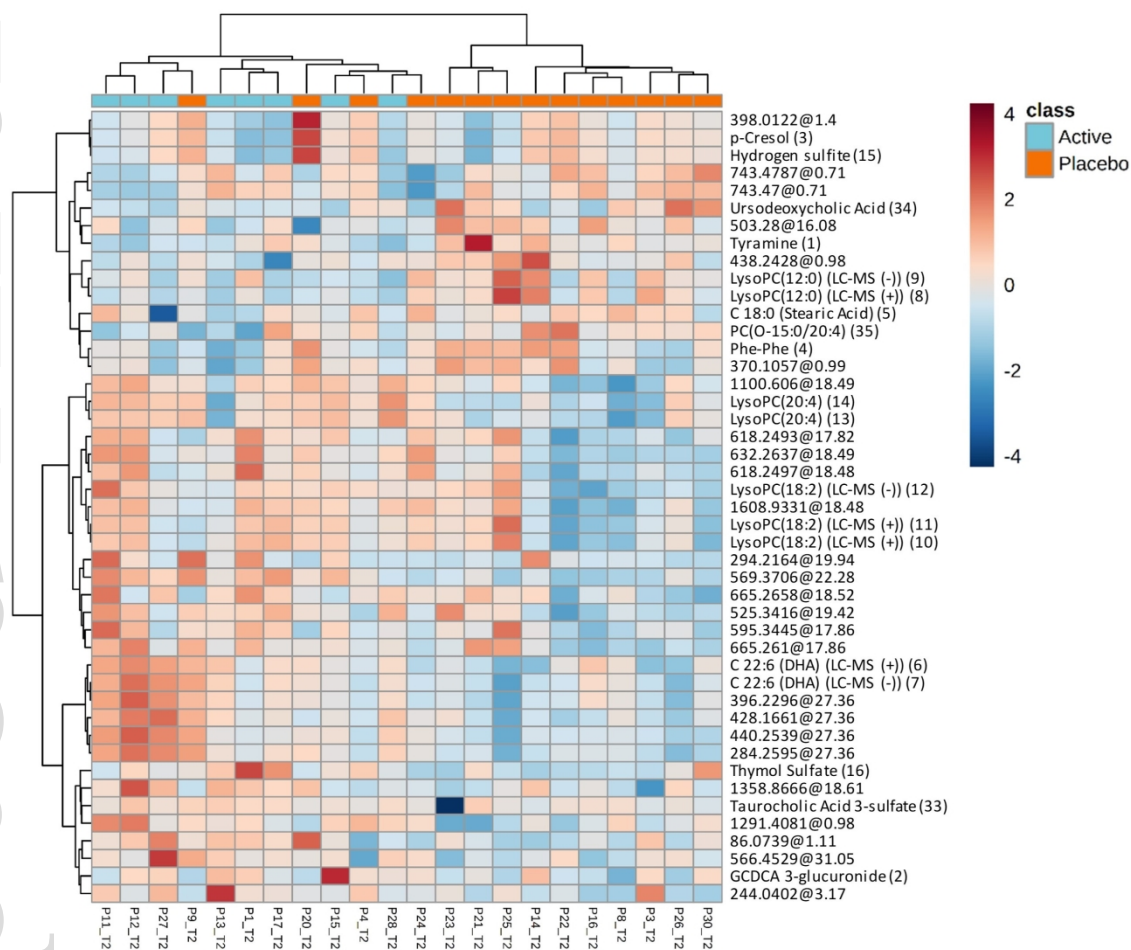


Figure 2

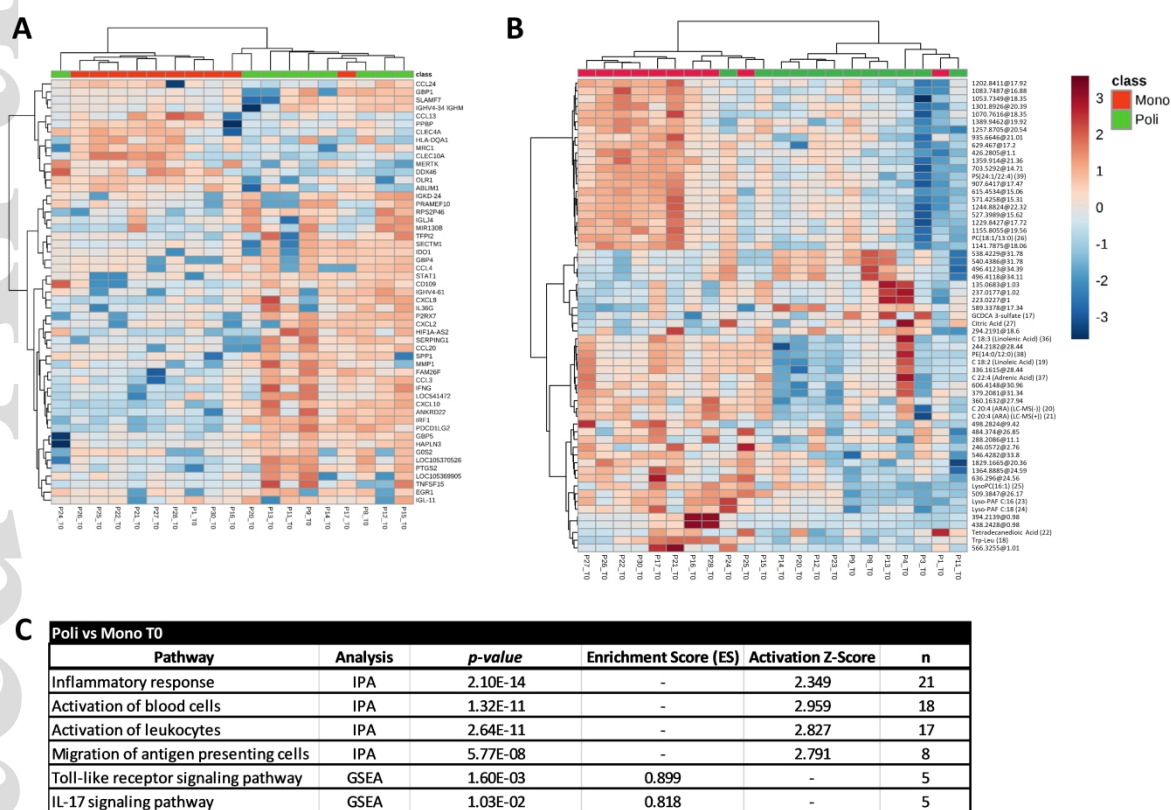
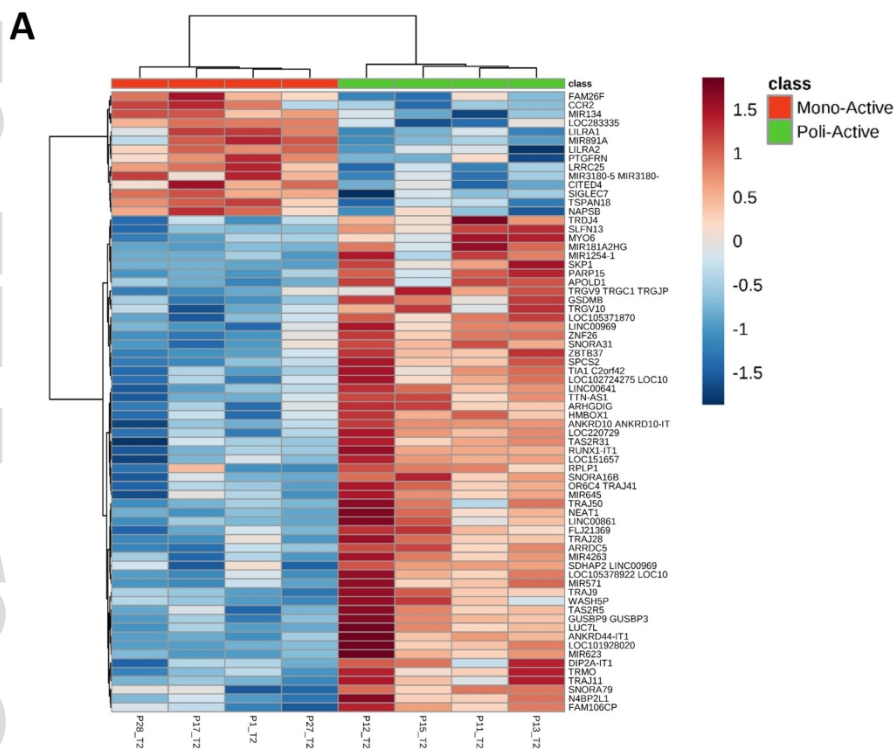


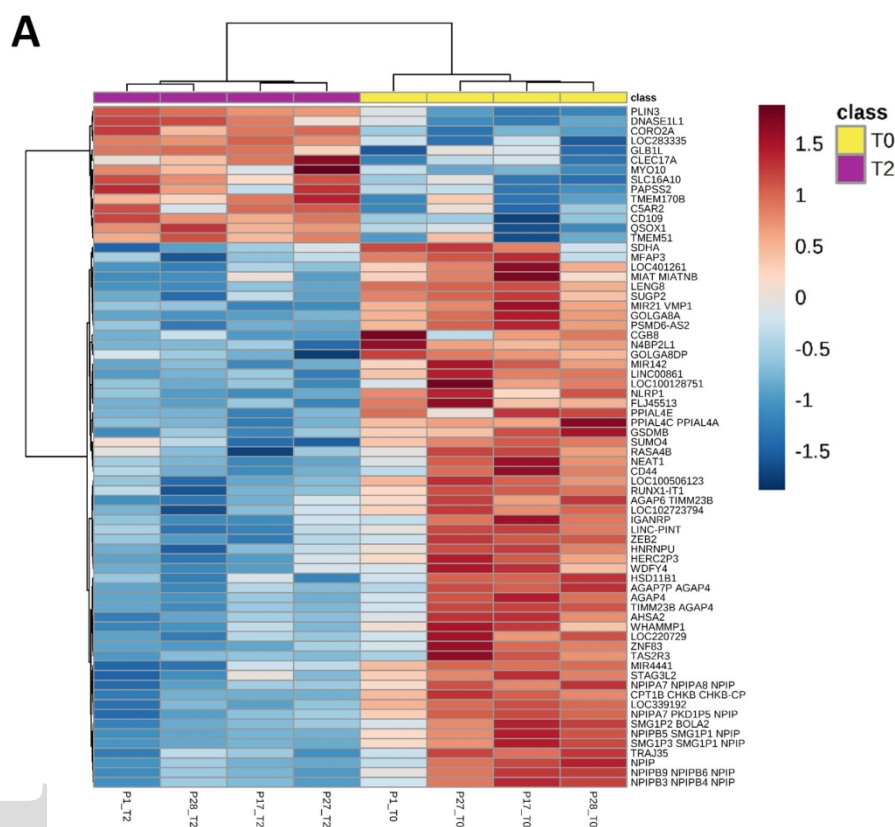
Figure 3



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



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

SUPPLEMENTARY INFORMATION

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

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1. PART 1. Detailed description of patient inclusion and study design

Forty-seven adult patients were enrolled by Hospital Universitario de La Princesa and Hospital Clínico Universitario San Carlos (Madrid, Spain) in this Phase IV exploratory randomized, parallel-group, double-blind, and placebo-controlled, 2-center national trial.

Subjects were randomized (1:1) during autumn 2013 to receive either active treatment with GRAZAX® (Phleum pratense, 75,000SQ-T tablets ALK, Hørsholm, Denmark) or placebo as Investigational Medical Products (IMP) during 2 years. Twenty-five patients were assigned to daily sublingual administration of GRAZAX®, while the rest received placebo tablets that were similar to the active IMP with regard to appearance, smell and taste.

The study consisted in 8 visits (V). In 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) (**Figure 1-I-A**). Thirty-one subjects remained at T2, from which 14 were Active and 17 Placebo (**Figure S-1**).

Main inclusion criteria were a clinical history of immunoglobulin E (IgE)-mediated grass pollen-induced rhinitis or rhinoconjunctivitis with or without asthma of at least one year prior to trial entry (2012 pollen season), a positive specific IgE against Phleum pratense (sIgE $\geq$ Class 2, $\geq$ 0.70 kU/l), and a positive skin prick test (SPT) (Soluprick SQ, ALK) response (wheal diameter $\geq$ 3 mm) to Phleum pratense.

Exclusion criteria included previous AIT, a clinical history of symptomatic perennial allergic rhinitis or perennial asthma, patients with contraindications for AIT as established by the immunotherapy subcommittee of the EAACI and by the Summary of Product Characteristics of GRAZAX®, and pregnancy. The institutional review board approved the study protocol; all subjects were informed of the aim of the trial and provided written consent.

2. PART 2. Detailed description of sample preparation, instrumental description, data pre-treatment and Quality Assurance for metabolomics analyses.

Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

Sample treatment

For LC-MS, proteins were removed adding 300 µL of cold (-20 °C) methanol:ethanol (1:1) to 100 µL of sample. Samples were then vortex-mixed and stored on ice for 5 min. Supernatant containing the metabolites was separated from the pellet by centrifugation (16,000× g for 20 min at 4 °C) and put into a LC vial for analysis.

Quality control (QC) Sample Preparation and randomization

QC samples were prepared by pooling equal volumes of plasma from each sample. The QC samples followed the same procedure applied for the experimental samples. In the case of LC-MS, one QC sample was used, whereas for GC-MS 8 QC samples were independently prepared. QC samples were analyzed throughout the run to provide a measurement of system stability, performance and the reproducibility of the sample treatment procedure. All samples prepared were randomized before metabolite extraction and for the corresponding analytical run.

LC-MS equipment

LC-MS analysis was performed on an Agilent HPLC system (1200 series, Agilent Technologies, Waldbronn, Germany), equipped with a degasser, two binary pumps, and a thermostated autosampler coupled with quadrupole-time of flight analyzer (Q-TOF) mass spectrometer (MS) series 6520 (Agilent Technologies).

Ten µL of extracted sample were injected into a Discovery HS C18 column (2.1 mm × 150 mm, 3.0 µm; Supelco, Sigma Aldrich, Germany), with a guard column Discovery® HS C18 (2 cm × 2.1 mm, 3 µm; Supelco, Sigma Aldrich, Germany), both maintained at 40 °C. The flow rate was set at 0.6 mL/min. The elution gradient involved a mobile phase consisting of: (A) 0.1% formic acid (FA) in water and (B) 0.1% FA in acetonitrile (ACN). The initial conditions were set at 25% phase B, which increased to 95% phase B in 35 min, followed by re-equilibration for 1 min and finally it was held for 9 min in initial conditions. The electrospray source ionization (ESI) data were acquired in both positive and negative ion modes. The

capillary voltage was set at 3,500 for ESI+ and 4,000V for ESI-, respectively. The drying gas flow rate was 10.5 L/min at 330 °C and gas nebulizer at 52 psi; fragmentor voltage was 175 V; skimmer and octopole radio frequency voltage (OCT RF Vpp) were set to 65 and 750 V, respectively. Data were collected in the centroid mode at a scan rate of 1.2 spectra per second. Mass spectrometry detection was performed in full scan from 100 to 1200 *m/z* for both positive and negative ESI modes. The reference *m/z* ions were purine (121.0508) and HP-0921 (922.0097) for ESI+, and TFA NH₄⁺ (119.0363) and HP-0921 (966.0007) for ESI-. These masses were continuously infused into the system to allow constant mass correction. Samples were analyzed in separate runs. These conditions were adapted from previous work.¹

Data treatment and compound identification

Acquired data were processed to provide structured raw data in an appropriate format for data analysis. Collected data were cleaned of background noises and unrelated ions using MassHunter Profinder (B.08.00, Agilent Technologies) software. Molecular feature extraction (MFE) algorithm was used to reduce the size and complexity of data. Furthermore, after MFE, Find by Ion (FBI) algorithm was applied to improve the reliability in finding the features in the data, obtaining 548 chemical signals for LC-MS positive ionization mode and 711 for negative ionization mode. Once the raw data was obtained, this was filtered keeping those features detected in >50% in QCs and with a Relative Standard Deviation (RSD) < 30%.

Metabolite annotation and LC-MS/MS experiments.

Database annotation was carried out for statistically significant features by searching the accurate masses against the online available databases such as KEGG, METLIN, LipidMaps and HMDB using an online advanced software called CEU Mass Mediator tool.² The annotation was done taking into account the retention time (RT) gradient, the isotopic pattern and possible adducts. The annotations were confirmed through LC-MS/MS experiments by using an LC-QTOF/MS equipment. Ions were targeted using the narrow *m/z* window (1.3 Da) and 20 V of energy for fragmentation on the quadrupole.

112

113 ***Gas Chromatography coupled to Mass Spectrometry (GC-MS)***

114 *Sample treatment*

115 The sample preparation methodology was adapted from previous publications.³ Serum samples were
116 first deproteinized using cold acetonitrile (ACN) in a 3:1 proportion (120 μ L of ACN were added to 40 μ L
117 of serum). Samples were kept on ice for 5 minutes. Afterwards, the metabolites were separated by
118 centrifugation (10 min at 15,400 x g and 4°C). 100 μ L of the resulting supernatant were transferred to a
119 GC vial with insert and were evaporated to dryness for 2 hours at 30°C (SpeedVac Concentrator, Thermo
120 Fisher Scientific, Waltham, MA, USA). Afterwards, a methoximation was performed by addition of 10 μ L
121 of O-methoxyamine hydrochloride 15mg/mL in pyridine, with the purpose of protecting the reactive
122 oxygen groups in the metabolites. The mixture was vigorously vortex-mixed (1 minute each vial),
123 ultrasonicated (3 times, 20 seconds each) and further vortexed another 2 minutes. Then, the samples
124 were left in darkness at room temperature for 16 h for the completion of the methoximation
125 reaction. Finally, the samples were derivatized by the addition of 10 μ L of N,O-
126 Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS).

127 This derivatization process seeks to add a trimethylsilyl group to the oxygen groups that were not
128 protected with the previous methoximation, a process named silylation which results in an increase of
129 the volatility of previously non-volatile compounds.

130 The vials were mixed vigorously and silylation was carried out by heating the samples in an oven for 1h
131 at 70 °C. The last step was the resuspension in 100 μ L of heptane containing 10 mg/L of an internal
132 standard (IS), in this case stearic acid (C18:0) methyl ester. All samples were vortex-mixed before GC
133 analysis.

134 3 blank samples were also prepared, following the same extraction and derivatization process but with
135 water instead of sample. In addition, QC samples were prepared. QCs were used to equilibrate the
136 equipment and to measure the variation of the analytical signal along the analysis. Afterwards they are
137 used to assess the analytical quality of the experiment. In this case, a sample pool was prepared by
138 mixing equal volumes of each sample before derivatization in an Eppendorf tube. Then, this pool was
139 distributed in 8 analytical vials and followed the same procedure as the experimental samples.

GC-Quadrupole-MS equipment and Analysis

Samples were measured in a gas chromatography system (GC, Agilent Technologies 7890A) equipped with an autosampler (Agilent 7693) and coupled to a mass spectrometer with an inert MSD quadrupole detector (Mass Selective Detector, Agilent 5975C). The specific parameters for the analysis are found in previously published literature.³ Two microliters (2 μ L) of the derivatized sample were injected through a DB5-MS GC-Column (30 m length, 0.25 mm i.d., 0.25 μ m film 95% dimethyl/5% diphenylpolysiloxane) with an integrated precolumn (10 m J&W, Agilent). Carrier gas (Helium) flow rate was set at 1 mL/min and injector temperature at 250 °C. Split ratio was fixed from 1:5 to 1:10 with 3 to 10 mL/min Helium split flow into a Restek 20782 (Bellefonte, PA, USA) deactivated glass-wool split liner. The temperature gradient was programmed as follows: the initial oven temperature was set at 60 °C (held for 1 min), increased to 325 °C at 10 °C/min rate (within 26.5 min) and was held at 325 °C for 10 min. The total run time was 37.5 min. A cool-down period of 10 min was applied before the next injection. Detector transfer line, filament source and quadrupole temperature were set at 280 °C, 230 °C and 150 °C, respectively. MS detection was performed with electron ionization (EI) mode at -70 eV. The mass spectrometer was operated in scan mode over a mass range of m/z 50-600 at a rate of 2.7 scan/s.

As for the analytical run, several IS injections were programmed at the start, followed by 2 injections of a mix of fatty acid methyl-esters (FAME C8-C30, Supelco, USA) which is used later to perform retention time correction for the FiehnLib library search. Afterwards, 3 QC samples were injected with the purpose of balancing the system. QC samples were also injected at regular intervals every 5 experimental samples, and 3 extraction blank injections were performed at the end of the run.

The positions of the experimental samples were randomized. These conditions were adapted from previous work.³

Data treatment and compound identification

GC-MS data, peak detection and spectra processing algorithms were applied using Agilent Mass Hunter Software (Agilent). The overall analytical performance was carefully examined by inspection of total ion chromatograms (TIC) of experimental samples, QC samples, blanks and internal standard. Raw data was

translated into a manageable extension file using GC-MS Translator software (MassHunter GC/MS Translator B.07.00, Agilent). Afterwards, automatic mass spectral deconvolution was employed to identify co-eluted compounds according to their retention indexes (RI) and RTs using the Unknowns Analysis software (B.08.00, Agilent). To achieve this, RTs derived from the analysis of FAME C8-C30 standard solution were used to create a calibration data file for further adjustment of RTs in samples. The improvement in accuracy was based on the determination of the Kovats RI. The RI value contained in Fiehn RTL (Retention Time Locked) library was compared to the experimental RI value to assign a match score between the experimental and the theoretical spectra. Missing compounds were identified by comparing their mass fragmentation patterns with NIST v.14 library (G1676AA, Agilent) using MassHunter Qualitative software (B.08.00, Agilent). Integration of the targets and alignment of the drift were performed on MassHunter Quantitative (B.08.00, Agilent). Raw data filtering was performed by removing those features detected in <50% of all QC samples. Data matrix was normalized by the IS to correct analytical variability of the equipment.

The mean abundance of each metabolite in the blank samples was calculated and subtracted from the abundance in the rest of the samples. As a last step, data was normalized using the QC-SVRC algorithm,⁴ as a way to minimize intra-group variance to maximize the potency of later statistical tests. Afterwards, the RSD was calculated, and compounds with values above 30% in the QCs were filtered.

3. PART 3. Detailed description of RNA extraction, microarray target preparation and hybridization for transcriptomic analysis.

RNA extraction: quantitation and integrity check

Total RNA from all the samples was isolated using RNeasy Mini Kit (QIAGEN) following manufacture procedure. RNA concentration was determined using a NanoDrop™ 2000/2000c Spectrophotometer. Then, its quality and integrity were assessed by Experion RNA StdSens Analysis Kit (Bio-Rad), selecting for downstream analysis those samples that had an RQI equal or higher than 7. Samples P3_T0, P4_T0, P23_T0, P3_T2, P4_T2 and P23_T2 did not satisfy quality requirements and were excluded from transcriptomic analysis.

Microarray target preparation and hybridization

Gene expression profile of the samples was analyzed using GeneChip Human Gene 2.1 ST strips (Affymetrix, Thermo Fisher Scientific) following GeneChip™ WT PLUS Reagent Kit manual instructions. Briefly, 100 ng of each sample were mixed with Poly-A RNA, then double-strand cDNA was synthesized from the starting RNA and cDNA was obtained by *in vitro* transcription (IVT) of the double stranded cDNA. Next, cDNA was used as template of sense-strand cDNA by reverse transcription and after that it was hydrolyzed, leaving only the single-stranded cDNA. Then, the single-stranded cDNA was fragmented by UDG and APE1 and labeled by TdT using DNA labeling Reagent that was covalently linked to biotin. Finally, the fragmented and labeled cDNA of all the samples was hybridized (4 samples for each strip) for 20 hours at 48°C and after that, the strips were washed, stained and imaged using GeneAtlas System (Thermo Fisher Scientific), acquiring CEL and DAT files.

Samples were analyzed as follows: 100 ng of RNA from each sample were mixed with Poly-A RNA. Then, double-strand cDNA was synthesized from the starting RNA and cDNA was obtained by *in vitro* transcription (IVT) of the double stranded cDNA. Next, cDNA was used as template of sense-strand cDNA by reverse transcription and after that it was hydrolyzed, leaving only the single-stranded cDNA. Then, the single-stranded cDNA was fragmented by UDG and APE1 and labeled by TdT using DNA labeling reagent that was covalently linked to biotin. Finally, the fragmented and labeled cDNA of all the samples was hybridized (4 samples for each strip) for 20 hours at 48°C and after that, the strips were washed, stained and imaged using GeneAtlas System (Thermo Fisher Scientific), acquiring CEL and DAT files.

Microarray data treatment for statistical analysis

Background correction, normalization, and summarization of probe-set level expression of the samples was performed applying the Robust Multi-array Average algorithm (RMA) method using Transcriptome Analysis Console 4.0 software (Affymetrix, Thermo Fisher Scientific).

Validation of results by qPCR

7 differentially expressed genes found with GSEA and IPA were selected for validation studies by quantitative PCR in order to confirm the results obtained with microarrays. For the RT-PCR the High Capacity RNA to cDNA Kit (Applied Biosystems) was used according to the manufacturer's instructions 1 µg of total RNA was reverse transcribed in a final volume of 20 µL.

225 Primers were designed using OligoArchitect™ (Sigma Aldrich) and qPCR was performed using SYBR
226 Green master mix (Takara) in the equipment Real Time HT 7900 (Applied Biosystems). Reactions were
227 run in triplicate. Expression data were normalized to the median of housekeeping gene β -actin and the
228 results were analyzed using the $2^{-\Delta\Delta CT}$ method.²⁰ 90% of selected genes showed the same expression
229 pattern as in the microarray experiments, so the results obtained can be considered reliable(**Figure S-3**).

SUPPLEMENTARY FIGURES AND TABLES

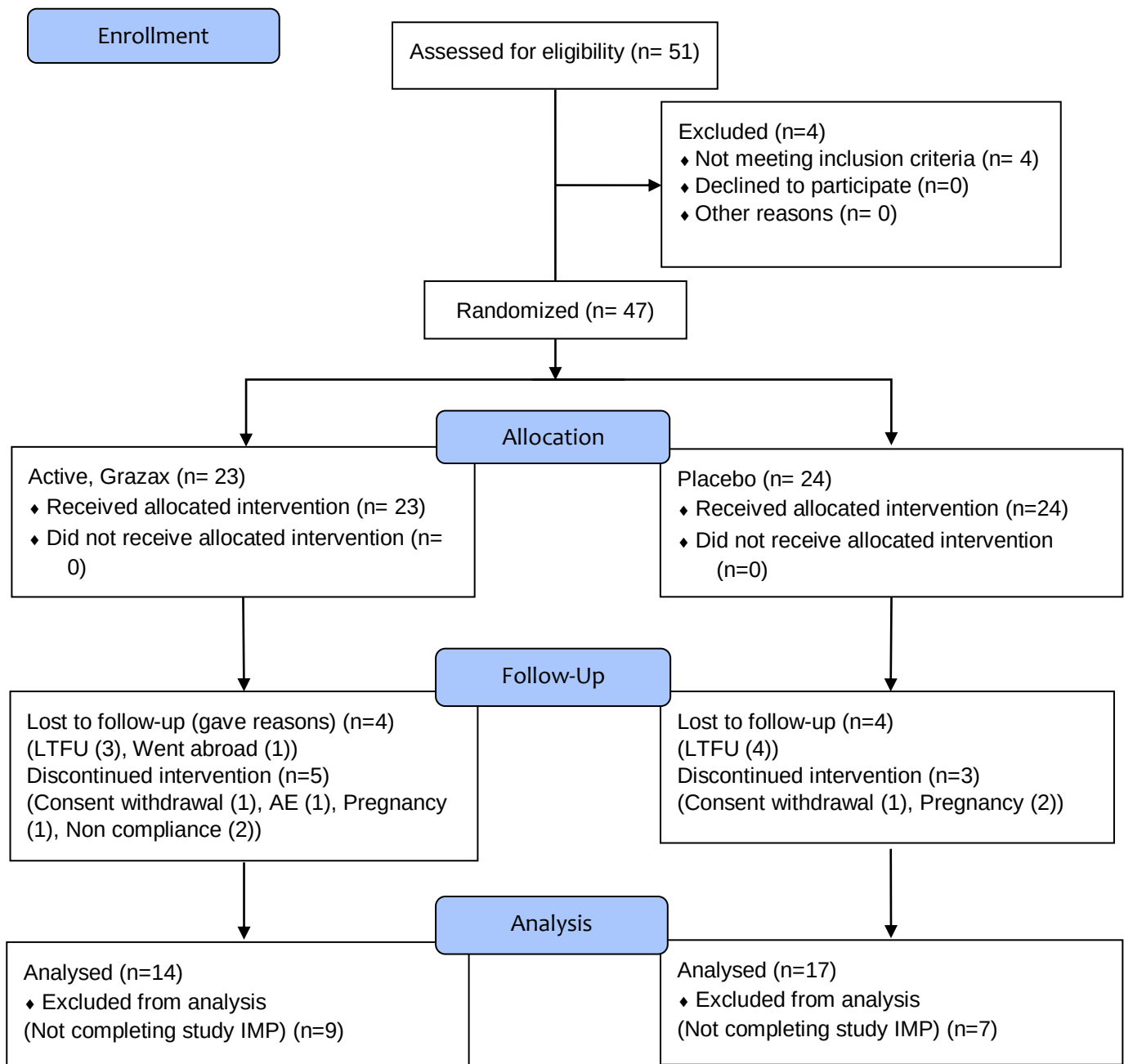


Figure S-1. Flow diagram: subject disposition. Adapted from consort-statement.org. NOTES: AE, adverse effects; IMP, investigational medical product.

259 **Table S-1.** Patients included in each comparison for omics analyses.

	Comparison	1st group			2nd group		
		Group	n	Patients	Group	n	Patients
Metabolomic Analysis	Active vs Placebo T2 (Fig.2)	Active T2	8	P1_T2, P11_T2, P12_T2, P13_T2, P15_T2, P17_T2, P27_T2, P28_T2	Placebo T2	14	P3_T2, P4_T2, P8_T2, P9_T2, P14_T2, P16_T2, P20_T2, P21_T2, P22_T2, P23_T2, P24_T2, P25_T2, P26_T2, P30_T2
	Poli vs Mono T0 (Fig. 3)	Poli T0	12	P3_T0, P4_T0, P8_T0, P9_T0, P11_T0, P12_T0, P13_T0, P14_T0, P15_T0, P20_T0, P23_T0, P24_T0	Mono T0	10	P1_T0, P16_T0, P17_T0, P21_T0, P22_T0, P25_T0, P26_T0, P27_T0, P28_T0, P30_T0
	Poli-Active vs Mono-Active T2	Poli-Active T2	4	P11_T2, P12_T2, P13_T2, P15_T2	Mono-Active T2	4	P1_T2, P17_T2, P27_T2, P28_T2
Transcriptomic Analysis	Poli vs Mono T0 (Fig. 3)	Poli T0	9	P8_T0, P9_T0, P11_T0, P12_T0, P13_T0, P14_T0, P15_T0, P20_T0, P24_T0	Mono T0	10	P1_T0, P16_T0, P17_T0, P21_T0, P22_T0, P25_T0, P26_T0, P27_T0, P28_T0, P30_T0
	Poli-Active vs Mono-Active T2 (Fig. 4)	Poli-Active T2	4	P11_T2, P12_T2, P13_T2, P15_T2	Mono-Active T2	4	P1_T2, P17_T2, P27_T2, P28_T2
	Mono-Active T2 vs Mono-Active T0 (Fig. 5)	Mono-Active T2	4	P1_T2, P17_T2, P27_T2, P28_T2	Mono-Active T0	4	P1_T0, P17_T0, P27_T0, P28_T0

261 **Table S-2.** Summary of clinical variables of the patients, *p*-values of their differences between groups, and test with which *p*-values were
262 obtained.t-test, Mann-Whitney, Chi-square (χ^2)or Fisher's exact test were applieddepending on the distribution and type of the datausing SPSS
263 (v. 24.0).†:Monosensitized to Phleum pretense, ‡ Polisenstized to Phleum pratense ± other pollens ± mites (animal dander are excluded).

264

	A) Including all patients (Serological kinetics analysis)			B) Excluding epithelial allergic patients (Omics analysis)			C) Comparing sensitization			
	ACTIVE	PLACEBO	<i>p</i> -value	ACTIVE	PLACEBO	<i>p</i> -value	MONO†	POLI‡	<i>p</i> -value	Test
Nº of subjects	14	17		8	14		10	12		
Mean age (SD) (years)	38 (10)	35 (7)	0.089	40 (13)	36 (8)	0.372	42 (9)	34 (6)	0.299	t-test
Female sex	7 (50%)	11 (65%)	0.409 ^a	3 (38%)	10 (71%)	0.187 ^b	6 (60%)	7 (58%)	1.000 ^b	a: Chi-square (χ^2) b: Fisher's exact test
Ethnicity			0.756 ^a			1.000 ^b			0.415 ^b	a: Chi-square (χ^2) b: Fisher's exact test
Caucasian	9 (64%)	10 (59%)		5 (62%)	8 (57%)		7 (70%)	6 (50%)		
Hispanic	5 (36%)	7 (41%)		3 (38%)	6 (43%)		3 (30%)	6 (50%)		
Phleum p5 (CAP class) (mode)	3	3	0.799	4	3	0.913	3	3-4	0.485	Mann-Whitney
Smoker (current or ex-smoker)	6 (43%)	4 (24%)	0.441	3 (38%)	4 (29%)	1.000	2 (20%)	5 (42%)	0.381	Fisher's exact test
Asthma	7 (50%)	10 (59%)	0.623 ^a	2 (25%)	8 (57%)	0.204 ^b	3 (30%)	7 (58%)	0.231 ^b	a: Chi-square (χ^2) b: Fisher's exact test
Sensitization (ISAC)			1.000			1.000				Fisher's exact test
Monosensitized to Phleum Pratense	4 (29%)	6 (35%)		4 (29%)	6 (35%)					
Polisenstized (pollens ± animal dander and/or mites)	10 (71%)	11 (65%)								
Polisenstized, excluding animal dander				4 (29%)	8 (47%)					
Allocation group									1.000	Fisher's exact test
Active							4 (40%)	4 (33,3%)		
Placebo							6 (60%)	8 (66,7%)		

265

Table S-3. Wilcoxon Signed Ranks Test p values for **A) slgE; B) slgG4; C) slgE/slgG4 ratio**. Cells in red represent statistically significant comparisons (p value < 0.05). M(0-24): 0 to 24 months of treatment; GPS: Grass Pollen Season.

A) slgE						
Time comparison	Active (n=14)	Placebo (n=17)	MONO-Active (n=4)	MONO-Placebo (n=6)	POLI-Active (n=4)	POLI-Placebo (n=8)
M1 - M4	0.004	0.001	0.144	0.027	0.068	0.017
M1 - GPSI	0.001	0.006	0.068	0.116	0.068	0.123
M1 - M12	0.001	0.522	0.068	0.833	0.068	0.123
M1 - GPSII	0.001	0.014	0.068	0.046	0.068	0.624
M1 - M24	0.001	0.705	0.068	0.116	0.068	0.327
M4 - GPSI	0.001	0.002	0.068	0.116	0.144	0.036
M4 - M12	0.001	0.028	0.068	0.249	0.068	0.310
M4 - GPSII	0.002	0.003	0.273	0.046	0.068	0.063
M4 - M24	0.001	0.068	0.068	0.075	0.068	0.484
GPSI - M12	0.003	0.008	0.068	0.046	0.068	0.025
GPSI - GPSII	0.006	0.813	0.465	0.528	0.068	0.263
GPSI - M24	0.001	0.006	0.144	0.249	0.068	0.069
M12 - GPSII	0.084	0.008	0.715	0.043	0.068	0.069
M12 - M24	0.005	1.000	0.273	0.116	0.068	0.726
GPSII - M24	0.005	0.001	0.068	0.028	0.465	0.025
B) slgG4						
Time comparison	Active (n=14)	Placebo (n=17)	MONO-Active (n=4)	MONO-Placebo (n=6)	POLI-Active (n=4)	POLI-Placebo (n=8)
M1 - M4	0.003	1.000	0.285	0.180	0.068	0.686
M1 - GPSI	0.003	0.062	0.144	0.593	0.068	0.173
M1 - M12	0.004	0.203	0.144	0.655	0.068	0.173
M1 - GPSII	0.003	0.028	0.068	0.655	0.068	0.046
M1 - M24	0.005	0.575	0.068	0.655	0.068	0.463
M4 - GPSI	0.048	0.010	0.144	0.285	0.465	0.046
M4 - M12	0.300	0.017	0.144	0.655	0.273	0.046
M4 - GPSII	0.272	0.025	0.068	0.655	0.144	0.075
M4 - M24	0.778	0.959	0.068	0.655	0.144	0.600
GPSI - M12	0.510	0.534	0.273	1.000	0.068	0.345
GPSI - GPSII	0.826	0.965	0.068	1.000	0.144	0.463
GPSI - M24	0.594	0.050	0.068	0.109	0.068	0.028
M12 - GPSII	0.397	0.359	0.068	0.655	0.465	0.833
M12 - M24	0.245	0.083	0.273	0.180	0.068	0.046
GPSII - M24	0.004	0.017	0.068	0.655	0.068	0.046
C) slgE/slgG4 ratio						
Time comparison	Active (n=14)	Placebo (n=17)	MONO-Active (n=4)	MONO-Placebo (n=6)	POLI-Active (n=4)	POLI-Placebo (n=8)
M1 - M4	0.001	0.002	0.068	0.058	0.068	0.025
M1 - GPSI	0.001	0.246	0.068	0.075	0.068	0.161
M1 - M12	0.001	0.246	0.068	0.462	0.068	0.036
M1 - GPSII	0.001	0.277	0.068	0.027	0.068	0.123
M1 - M24	0.001	0.906	0.068	0.116	0.068	0.161
M4 - GPSI	0.001	0.014	0.068	0.115	0.068	0.362
M4 - M12	0.005	0.523	0.066	0.249	0.066	0.262
M4 - GPSII	0.001	0.013	0.068	0.028	0.068	0.735
M4 - M24	0.001	0.031	0.068	0.116	0.068	0.326
GPSI - M12	0.102	0.002	0.068	0.046	0.068	0.068
GPSI - GPSII	0.023	0.758	0.109	0.345	0.109	0.944
GPSI - M24	0.030	0.163	0.068	0.345	0.068	0.889
M12 - GPSII	0.102	0.017	0.465	0.028	0.465	0.208
M12 - M24	0.054	0.055	0.144	0.116	0.144	0.012
GPSII - M24	0.556	0.023	0.715	0.028	0.715	0.866

272 **Table S-4.A)** sIgE and B) sIgG4 values for active and placebo patients during treatment.

Table S-4 A) sIgE (kUA/L)									
Time			0M	1M	4M	GPS I	12M	GPS II	24M
Patient	Treatment	Sensitization							
1	Active	Mono	19.75	235.04	127.75	74.58	48.24	30.40	23.58
2	Active	Poli-Epi	9.41	55.39	27.44	19.80	14.07	6.91	6.20
5	Active	Poli-Epi	65.83	507.98	374.53	230.04	197.66	183.11	177.87
6	Active	Poli-Epi	24.72	60.21	61.95	42.94	52.66	21.61	17.66
10	Active	Poli-Epi	17.98	67.88	70.01	62.97	42.40	34.42	31.44
11	Active	Poli	21.43	304.82	117.42	59.92	33.69	19.55	13.40
12	Active	Poli	126.92	470.84	382.78	394.37	371.49	278.88	311.00
13	Active	Poli	41.88	105.46	101.38	70.55	46.96	35.01	30.62
15	Active	Poli	14.66	24.11	21.51	13.97	9.48	7.31	6.42
17	Active	Mono	17.80	67.22	68.08	54.91	43.23	59.99	35.77
18	Active	Poli-Epi	32.71	71.73	52.05	45.58	34.20	25.33	23.54
27	Active	Mono	1.21	23.37	16.29	14.61	8.82	7.20	4.42
28	Active	Mono	35.74	111.79	54.49	34.48	20.42	76.77	46.77
29	Active	Poli-Epi	39.17	264.01	207.27	168.56	68.48	54.55	29.65
3	Placebo	Poli	25.05	26.14	18.34	46.42	23.10	27.37	20.26
4	Placebo	Poli	23.78	17.29	17.96	18.28	19.90	18.01	20.65
7	Placebo	Poli-Epi	36.08	28.98	22.17	190.60	237.42	206.91	143.34
8	Placebo	Poli	182.31	150.09	141.90	270.76	179.18	198.94	144.54
9	Placebo	Poli	65.67	59.68	43.79	83.21	54.57	135.86	110.55
14	Placebo	Poli	37.24	46.46	28.71	29.31	27.36	21.72	20.47
16	Placebo	Mono	25.48	23.14	20.03	19.76	21.29	44.01	31.67
19	Placebo	Poli-Epi	187.42	162.63	105.48	246.28	155.46	383.10	136.96
20	Placebo	Poli	5.49	4.98	4.06	7.73	4.05	6.02	4.41
21	Placebo	Mono	11.23	13.59	10.60	28.49	16.46	29.58	18.75
22	Placebo	Mono	27.57	19.01	13.51	34.22	19.69	49.06	27.05
23	Placebo	Poli	20.52	21.78	14.54	27.52	18.86	20.81	17.82
24	Placebo	Poli	6.09	5.89	4.72	4.35	3.34	4.70	3.63
25	Placebo	Mono	0.83	0.60	0.44	2.80	0.72	1.42	0.56
26	Placebo	Mono	12.48	12.06	11.90	11.49	10.31	10.36	9.17
30	Placebo	Mono	2.54	2.24	2.11	2.69	1.92	5.01	3.10
31	Placebo	Poli-Epi	1.30	1.18	1.31	1.93	1.25	1.18	0.95

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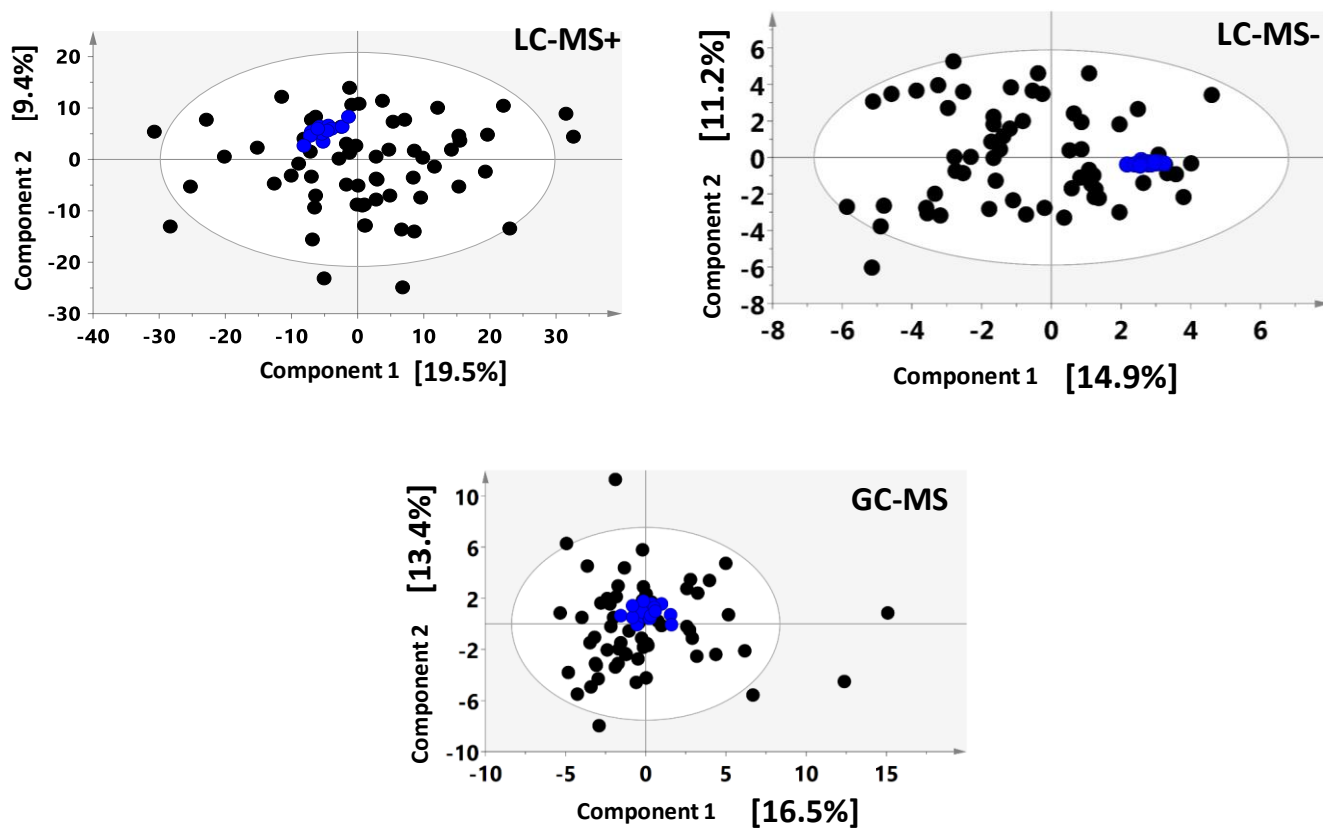
274 **Legend:** Mono: Monosensitized patients; Poli: Polisensitized; Poli-Epi: Polisensitized patients with epithelial allergy; (0-24)M: 0 to 24 months of
 275 treatment; GPS: Grass Pollen Season.

276

Table S-4 B) sIgG4 (mgA/L)									
Time			0M	1M	4M	GPS I	12M	GPS II	24M
Patient	Treatment	Sensitization							
1	Active	Mono	0.08	0.09	0.42	0.58	0.75	1.30	0.95
2	Active	Poli-Epi	0.13	0.43	1.05	2.63	4.97	4.29	3.78
5	Active	Poli-Epi	0.43	2.72	2.62	1.99	1.11	1.25	1.52
6	Active	Poli-Epi	0.08	0.11	0.34	0.49	0.48	0.46	0.38
10	Active	Poli-Epi	0.08	0.08	0.12	0.20	0.17	0.10	0.08
11	Active	Poli	0.14	1.01	2.28	3.85	1.87	1.96	1.60
12	Active	Poli	0.08	0.08	0.29	0.35	0.32	0.39	0.32
13	Active	Poli	0.08	0.08	1.22	0.76	0.39	0.51	0.26
15	Active	Poli	0.08	0.09	0.37	0.44	0.35	0.24	0.16
17	Active	Mono	0.08	0.29	0.53	0.60	0.60	0.65	0.64
18	Active	Poli-Epi	0.11	0.40	0.55	0.60	0.99	0.95	1.01
27	Active	Mono	0.08	0.08	0.08	0.13	0.22	0.29	0.17
28	Active	Mono	0.08	0.28	0.27	0.26	0.24	0.74	0.52
29	Active	Poli-Epi	0.08	0.18	0.42	0.68	0.52	0.46	0.24
3	Placebo	Poli	0.16	0.17	0.31	0.37	0.43	0.30	0.31
4	Placebo	Poli	0.08	0.08	0.08	0.08	0.08	0.08	0.08
7	Placebo	Poli-Epi	0.08	0.08	0.11	0.23	0.33	0.49	0.36
8	Placebo	Poli	0.24	0.24	0.37	0.71	0.50	0.49	0.35
9	Placebo	Poli	0.32	0.24	0.23	0.38	0.33	0.41	0.36
14	Placebo	Poli	0.19	0.15	0.11	0.19	0.16	0.11	0.09
16	Placebo	Mono	0.08	0.08	0.08	0.08	0.08	0.08	0.08
19	Placebo	Poli-Epi	0.21	0.41	0.31	0.39	0.33	0.43	0.24
20	Placebo	Poli	0.20	0.37	0.31	0.25	0.26	1.21	0.21
21	Placebo	Mono	0.08	0.08	0.08	0.09	0.08	0.08	0.08
22	Placebo	Mono	0.08	0.08	0.08	0.08	0.08	0.08	0.08
23	Placebo	Poli	0.08	0.08	0.08	0.12	0.10	0.10	0.08
24	Placebo	Poli	0.08	0.08	0.08	0.08	0.08	0.08	0.08
25	Placebo	Mono	0.08	0.08	0.08	0.11	0.11	0.15	0.10
26	Placebo	Mono	0.08	0.14	0.13	0.13	0.13	0.11	0.12
30	Placebo	Mono	0.08	0.08	0.08	0.08	0.08	0.08	0.08
31	Placebo	Poli-Epi	0.08	0.08	0.08	0.08	0.08	0.08	0.08

277

278 **Legend:** Mono: Monosensitized patients; Poli: Polisensitized; Poli-Epi: Polisensitized patients with epithelial allergy; (0-24)M: 0 to 24 months of
279 treatment;GPS: Grass Pollen Season.



280

281 **Figure S-2.** Principal Component Analysis (PCA) models for the raw data from the three analytical
 282 techniques used in the study. Quality Control (QC) samples are highlighted in blue.

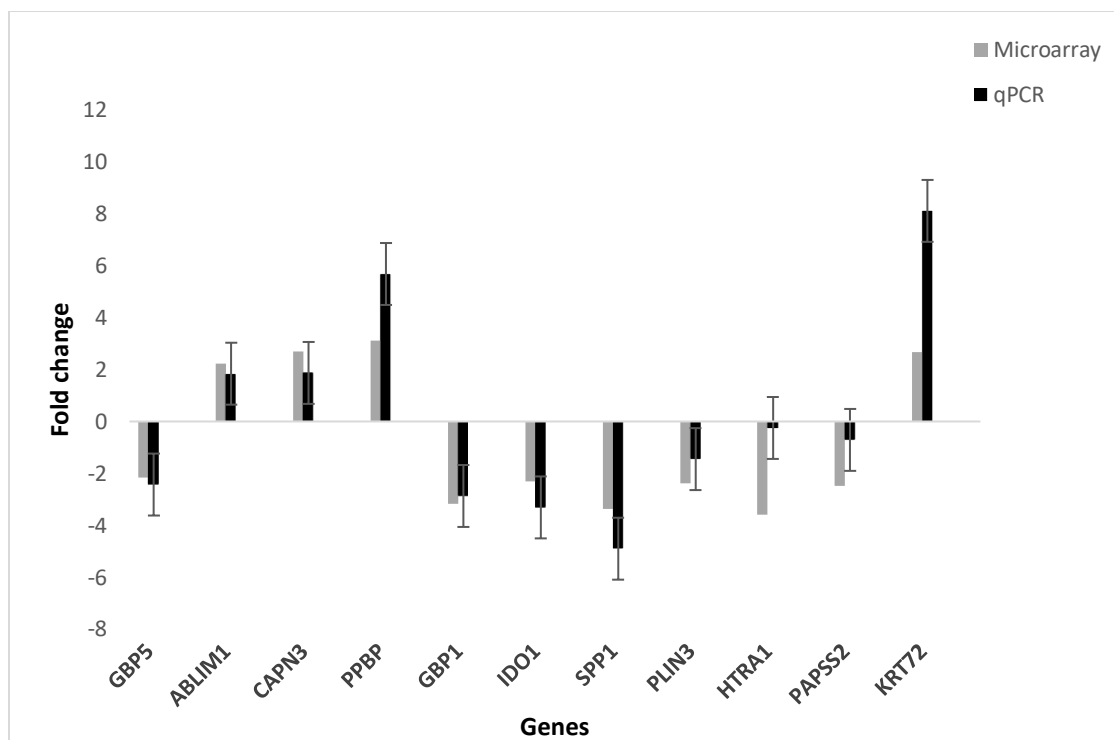


Figure S-3. Validation of results by qPCR. Microarrays results (grey) are shown compared to qPCR results (black). Standard deviation bars are represented in qPCR results

Table S-5. Changes in the significant annotated metabolites in the three comparisons.				(A) Active vs Placebo T2			(B) Poli vs Mono T0			(C) Poli-Active vs Mono-Active T2		
Nº	Technique	Compound Annotation	Biological Class	p	p BH	%Δ in Active	p	p BH	%Δ in Poli	p	p BH	%Δ in Poli
1	LC-MS (pos)	Tyramine	Amino acid derivatives	0.0441	0.0441	-13%						
2	LC-MS (neg)	GCDCA 3-glucuronide	Bile acids	0.0185	0.0441	72%						
3	LC-MS (neg)	p-Cresol	Xenobiotics	0.0441	0.0441	-45%						
4	LC-MS (pos)	Phe-Phe	Dipeptides	0.0441	0.0441	-28%						
5	GC-MS	C 18:0 (Stearic Acid)	Fatty acids	0.0441	0.0441	-19%						
6	LC-MS (pos)	C 22:6 (DHA)	Fatty acids	0.0154	0.0424	43%						
7	LC-MS (neg)	C 22:6 (DHA)	Fatty acids	0.0070	0.0292	39%						
8	LC-MS (pos)	LysoPC(12:0)	Glycerophospholipids	0.0127	0.0424	-51%						
9	LC-MS (neg)	LysoPC(12:0)	Glycerophospholipids	0.0316	0.0441	-43%						
10	LC-MS (pos)	LysoPC(18:2)	Glycerophospholipids	0.0316	0.0424	21%						
11	LC-MS (pos)	LysoPC(18:2)	Glycerophospholipids	0.0316	0.0424	19%						
12	LC-MS (neg)	LysoPC(18:2)	Glycerophospholipids	0.0441	0.0441	19%						
13	LC-MS (pos)	LysoPC(20:4)	Glycerophospholipids	0.0265	0.0424	24%						
14	LC-MS (pos)	LysoPC(20:4)	Glycerophospholipids	0.0374	0.0424	21%						
15	LC-MS (neg)	Hydrogen sulfite	Sulfites	0.0265	0.0441	-44%						
16	LC-MS (neg)	Thymol Sulfate	Xenobiotics	0.0222	0.0441	193%						
17	LC-MS (neg)	GCDCA 3-sulfate	Bile acids				0.0443	0.0443	57%			
18	LC-MS (pos)	Trp-Leu	Dipeptides				0.0092	0.0443	-79%			
19	LC-MS (pos)	C 18:2 (Linoleic Acid)	Fatty acids				0.0321	0.0443	-31%			
20	LC-MS (neg)	C 20:4 (Arachidonic Acid, AA)	Fatty acids				0.0378	0.0443	-27%			
21	LC-MS (pos)	C 20:4 (Arachidonic Acid, AA)	Fatty acids				0.0443	0.0443	-30%			
22	LC-MS (neg)	Tetradecanedioic Acid	Fatty acids				0.0192	0.0443	-46%			
23	LC-MS (pos)	Lyso-PAF C:16	Glycerophospholipids				0.0378	0.0443	-20%			
24	LC-MS (pos)	Lyso-PAF C:18	Glycerophospholipids				0.0272	0.0443	-17%			
25	LC-MS (neg)	LysoPC(16:1)	Glycerophospholipids				0.0378	0.0443	-22%			
26	LC-MS (pos)	PC(18:1/13:0)	Glycerophospholipids				0.0111	0.0443	-19%			
27	GC-MS	Citric Acid	Tricarboxylic acids				0.0134	0.0134	100%			
28	GC-MS	Trans-4-hydroxy-L-Proline	Amino acids							0.0286	0.0286	86%
29	GC-MS	Levogluconan	Anhydrohexoses							0.0286	0.0286	-78%
30	GC-MS	Benzoic Acid	Xenobiotics							0.0286	0.0286	46%
31	LC-MS (neg)	Deoxycholic Acid 3-glucuronide	Bile acids							0.0286	0.0286	-33%
32	GC-MS	Erythritol	Sugar alcohols							0.0286	0.0286	-71%
33	LC-MS (neg)	Taurocholic Acid 3-sulfate	Bile acids	0.0222	0.0441	20%						
34	LC-MS (neg)	Ursodeoxycholic Acid	Bile acids	0.0441	0.0441	-59%						
35	LC-MS (pos)	PC(O-15:0/20:4)	Glycerophospholipids	0.0316	0.0424	-9%						
36	LC-MS (neg)	C 18:3 (Linolenic Acid)	Fatty acids				0.0229	0.0443	-22%			
37	LC-MS (neg)	C 22:4 (Adrenic Acid)	Fatty acids				0.0378	0.0443	-17%			
38	LC-MS (neg)	PE(14:0/12:0)	Glycerophospholipids				0.0443	0.0443	-25%			
39	LC-MS (pos)	PS(24:1/22:4)	Glycerophospholipids				0.0192	0.0443	-20%			
40	LC-MS (pos)	3-Keto-Sphingosine	Sphingolipid							0.0286	0.0286	161%

Notes: **p**:*p*-value. **p BH**:*p*-value with Benjamini-Hochberg correction. **%Δ**: percentage of change (negative values are highlighted in red and positive values in green).**AA**: Arachidonic acid.**DHA**: Docosahexaenoic acid.**GCDCA**: Glycochenodeoxycholic Acid.**LysoPC**: Lysophosphatidylcholine. **PC**: Phosphatidylcholine. **PE**: Phosphatidylethanolamine. **PS**: Phosphatidylserine. **PAF**: Platelet-activating factor. **“Poli”** are Poli-sensitized patients (excluding epithelial allergic patients) before treatment; **“Mono”** are Mono-sensitized patients before treatment; **“Poli-Active”** are Poli-sensitized patients (excluding epithelial allergic patients) after 2 years of Active; **“Mono-Active”** are Mono-sensitized patients after 2 years of Active. The specific patients included in each analysis are shown in **Table S-1**.

Table S-6: Physicochemical properties and analytical parameters of significant metabolites from all comparisons in the study and all three analytical techniques.											
Nº	Technique	Compound Annotation	Formula	Biochemical Class	Adduct	RT (min)	Experimental Mass (Da)	m/z	Mass Error (ppm)	Confidence Level	CV % in QCs
1	LC-MS (pos)	Tyramine	C8H11NO	Amino acid derivatives	M+H-H2O	0.99	137.0834	120.0801	-5.11	MS/MS	5%
2	LC-MS (neg)	GCDCA3-glucuronide	C32H51NO11	Bile acids	M-H	8.29	625.3464	624.3392	0.32	MS/MS	8%
3	LC-MS (neg)	p-Cresol	C7H8O	Xenobiotics	M-H	1.41	108.0578	107.0506	2.78	MS/MS	2%
4	LC-MS (pos)	Phe-Phe	C18H20N2O3	Dipeptides	M+H	0.98	312.1477	313.1549	0.96	MS/MS	8%
5	GC-MS	C 18:0 (Stearic Acid)	C18H36O2	Fatty acids	-	20.68	-	341	-	Fiehn + NIST	28%
6	LC-MS (pos)	C 22:6 (DHA)	C22H32O2	Fatty acids	M+H	27.34	328.2417	329.2489	4.57	MS/MS	11%
7	LC-MS (neg)	C 22:6 (DHA)	C22H32O2	Fatty acids	M-H	27.36	328.2399	327.2327	-0.91	MS/MS	4%
8	LC-MS (pos)	LysoPC(12:0)	C20H42NO7P	Glycerophospholipids	M+H	12.06	439.2706	440.2778	1.59	MS/MS	9%
9	LC-MS (neg)	LysoPC(12:0)	C20H42NO7P	Glycerophospholipids	M+FA-H	12.09	439.2720	484.2702	4.78	MS/MS	8%
10	LC-MS (pos)	LysoPC(18:2)	C26H50NO7P	Glycerophospholipids	M+H	18.48	519.333	520.3402	0.96	MS/MS	8%
11	LC-MS (pos)	LysoPC(18:2)	C26H50NO7P	Glycerophospholipids	M+H	17.83	519.3332	520.3404	1.35	MS/MS	9%
12	LC-MS (neg)	LysoPC(18:2)	C25H48NO7P	Glycerophospholipids	M-CH3-H	18.52	505.3168	504.3098	0.00	MS/MS	12%
13	LC-MS (pos)	LysoPC(20:4)	C28H50NO7P	Glycerophospholipids	M+H	17.93	543.3326	544.3398	0.18	MS/MS	16%
14	LC-MS (pos)	LysoPC(20:4)	C28H50NO7P	Glycerophospholipids	M+H	18.50	543.3329	544.3401	0.74	MS/MS	13%
15	LC-MS (neg)	Hydrogen sulfite	HO3S	Sulfites	M-H	1.41	80.9650	79.9578	4.94	MS/MS	4%
16	LC-MS (neg)	Thymol Sulfate	C10H14O4S	Xenobiotics	M-H	5.41	230.0612	229.0540	-0.43	MS/MS	6%
17	LC-MS (neg)	GCDCA3-sulfate	C26H43NO8S	Bile acids	M-H	9.08	529.2708	528.2636	-0.19	MS/MS	23%
18	LC-MS (pos)	Trp-Leu	C17H23N3O3	Dipeptides	M+H	0.99	317.1803	318.1875	20.18	MS/MS	8%
19	LC-MS (pos)	C 18:2 (Linoleic Acid)	C18H32O2	Fatty acids	M+H	28.44	280.2406	281.2478	1.43	MS/MS	11%
20	LC-MS (neg)	C 20:4 (Arachidonic Acid, AA)	C20H32O2	Fatty acids	M-H	27.97	304.2403	303.2331	0.33	MS/MS	4%
21	LC-MS (pos)	C 20:4 (Arachidonic Acid, AA)	C20H32O2	Fatty acids	M+H	27.94	304.2428	305.2500	8.55	MS/MS	13%
22	LC-MS (neg)	Tetradecanedioic Acid	C14H26O4	Fatty acids	M-H	11.69	258.1834	257.1762	1.16	MS/MS	16%
23	LC-MS (pos)	Lyso-PAF C:16	C24H52NO6P	Glycerophospholipids	M+H	21.37	481.3528	482.3600	-0.83	MS/MS	8%
24	LC-MS (pos)	Lyso-PAF C:18	C26H54NO6P	Glycerophospholipids	M+H	22.18	507.3686	508.3758	-0.59	MS/MS	7%
25	LC-MS (neg)	LysoPC(16:1)	C24H48NO7P	Glycerophospholipids	M+FA-H	18.26	493.3182	538.3164	2.84	MS/MS	7%
26	LC-MS (pos)	PC(18:1/13:0)	C39H76NO8P	Glycerophospholipids	M+H	16.58	717.5272	718.5344	-5.16	MS/MS	11%
27	GC-MS	Citric Acid	C6H8O7	Tricarboxylic acids	-	16.53	-	273	-	Fiehn + NIST	4%
28	GC-MS	Trans-4-hydroxy-L-Proline	C5H9NO3	Amino acids	-	12.574, 13.2	-	158, 230	-	Fiehn + NIST	12%
29	GC-MS	Levoglucosan	C6H10O5	Anhydrohexoses	-	15.276	-	204	-	Fiehn + NIST	28%
30	GC-MS	Benzoic Acid	C7H6O2	Xenobiotics	-	9.565	-	179	-	Fiehn + NIST	9%
31	LC-MS (neg)	Deoxycholic Acid 3-glucuronide	C30H48O10	Bile acids	M-H	11.2	568.3250	567.3178	0.53	MS/MS	5%
32	GC-MS	Erythritol	C4H10O4	Sugar alcohols	-	12.85	-	217	-	Fiehn + NIST	5%
33	LC-MS (neg)	Taurocholic Acid 3-sulfate	C26H45NO10S2	Bile acids	M+FA-H	20.15	595.2579	640.2561	15.79	Tentative	13%
34	LC-MS (neg)	Ursodeoxycholic Acid	C24H40O4	Bile acids	M-H	10.89	392.2916	391.2844	-2.80	Tentative	2%
35	LC-MS (pos)	PC(O-15:0/20:4)	C42H82NO9P	Glycerophospholipids	M+H	18.25	775.5641	776.5713	-11.09	Tentative	14%
36	LC-MS (neg)	C 18:3 (Linolenic Acid)	C18H30O2	Fatty acids	M-H	25.97	278.2243	277.2171	-1.08	Tentative	4%
37	LC-MS (neg)	C 22:4 (Adrenic Acid)	C22H36O2	Fatty acids	M-H	30.79	332.2710	331.2638	-1.50	Tentative	5%
38	LC-MS (neg)	PE(14:0/12:0)	C31H62NO8P	Glycerophospholipids	M+Cl	28.46	607.4283	642.3977	11.52	Tentative	7%
39	LC-MS (pos)	PS(24:1/22:4)	C52H92NO10P	Glycerophospholipids	M+H	19.20	921.6602	922.6674	15.52	Tentative	14%
40	LC-MS (pos)	3-Keto-Sphingosine	C18H35NO2	Sphingolipid	M+Na	17.34	297.2668	320.2542	8.07	Tentative	7%

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