

ORIGINAL ARTICLE

Experimental Allergy and Immunology

Multi-omics analysis points to altered platelet functions in severe food-associated respiratory allergy

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Abstract

Background: Prevalence and severity of allergic diseases have increased worldwide. To date, respiratory allergy phenotypes are not fully characterized and, along with inflammation progression, treatment is increasingly complex and expensive. Profilin sensitization constitutes a good model to study the progression of allergic inflammation. Our aim was to identify the underlying mechanisms and the associated biomarkers of this progression, focusing on severe phenotypes, using transcriptomics and metabolomics.

Methods: Twenty-five subjects were included in the study. Plasma samples were analyzed using gas and liquid chromatography coupled to mass spectrometry (GC-MS and LC-MS, respectively). Individuals were classified in four groups—“nonallergic,” “mild,” “moderate,” and “severe”—based on their clinical history, their response to an oral challenge test with profilin, and after a refinement using a mathematical metabolomic model. PBMCs were used for microarray analysis.

Abbreviations: ARA, arachidonic acid; 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, lysophosphatidylcholines; MS/MS, tandem mass spectrometry; PBMCs, peripheral blood mononuclear cells; QC, quality control; RT, retention time; S1P, sphingosine-1-phosphate.

Obeso and Mera-Berriatua equally contributed to this work.
 Villaseñor and Escribese equally contributed to this work.

Results: We found a set of transcripts and metabolites that were specific for the “severe” phenotype. By metabolomics, a decrease in carbohydrates and pyruvate and an increase in lactate were detected, suggesting aerobic glycolysis. Other metabolites were incremented in “severe” group: lysophospholipids, sphingosine-1-phosphate, sphinganine-1-phosphate, and lauric, myristic, palmitic, and oleic fatty acids. On the other hand, carnitines were decreased along severity. Significant transcripts in the “severe” group were found to be downregulated and were associated with platelet functions, protein synthesis, histone modification, and fatty acid metabolism.

Conclusion: We have found evidence that points to the association of severe allergic inflammation with platelet functions alteration, together with reduced protein synthesis, and switch of immune cells to aerobic glycolysis.

KEYWORDS

food allergy, metabolomics, platelets, respiratory allergy, transcriptomics

1 | INTRODUCTION

Allergic diseases affect more than a quarter of the population and present an increasing trend. It is possible that in a near future, economic impact of allergy might reach a unsustainable cost. Consequently, it is pivotal to unravel the mechanisms involved in this process in order to develop new diagnostic, intervention, and prevention strategies. However, one of the main problems found in the quest for these new strategies is the difficulty of having properly clinically stratified patients that can be used for the development of accurate models.

In the last years, component-resolved diagnosis strategies¹ have allowed a significant increase in the understanding of allergic disease evolution. In this context, molecular-based epidemiological studies performed in Spain^{2–4} allowed the identification of different clinical phenotypes of the dominant pollen allergies (olive and grass pollen). In areas of extreme pollen exposure, patients often display a more severe phenotype.⁵ In fact, some grass pollen-allergic patients resident in highly exposed regions can develop severe food allergic reactions mediated by profilin,⁶ a type of reaction hardly seen in other grass pollen-exposed territories. Profilin has proved to be a clinically relevant aeroallergen^{7,8} able to induce strong T-cell proliferation.⁹ Recently, it has been proved that evolution toward severity in the profilin food allergy model is associated with a progressive rise in effector cell sensitivity, immune cells infiltration in the epithelia, and structural remodeling of the oral mucosa.¹⁰ This provides evidence that oral mucosa may play a key role in both food allergic reactions and respiratory-to-food allergy evolution. Moreover, severe profilin reactors express relatively high levels of periostin, a biomarker linked to airway inflammation and remodeling.¹¹

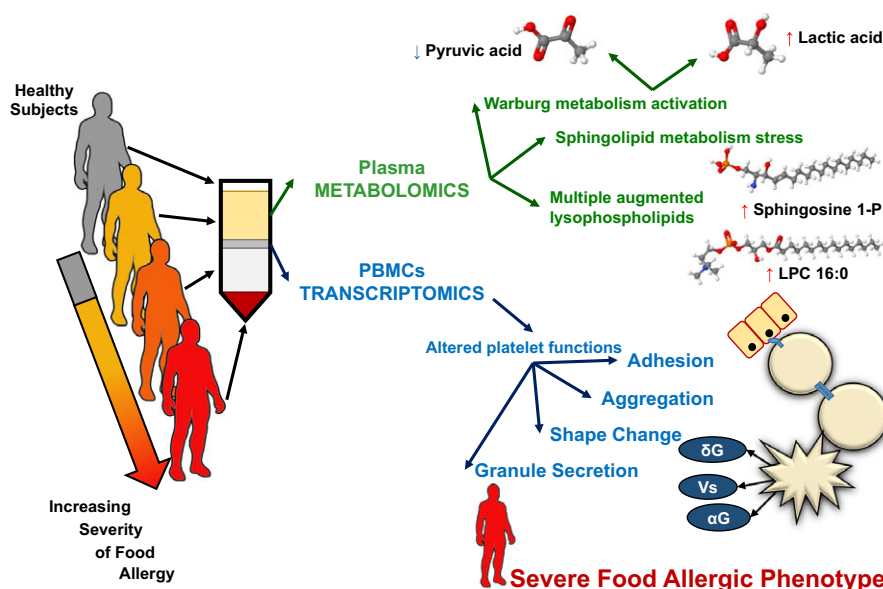
As a consequence, the group of severe reactors to profilin provides a unique clinical model for understanding allergy progression and associated barrier inflammation. Moreover, by mean of integrated –omics approaches, new potential diagnostic strategies connected with progressive allergic endo-phenotypes can be uncovered. This is a clear unmet need in allergy research.¹²

The –omics sciences have emerged as a new strategy to study living organisms. They offer the possibility of a better understanding of a disease by obtaining vast amounts of data from every individual biological sample. Transcriptomics, and to a lesser extent metabolomics, have been previously used to uncover the molecular mechanisms involved in the development of several allergic diseases.^{13–20} Previous studies have not yet addressed neither specific allergic phenotypes nor their associated metabolic signatures, and these are still largely unknown. Therefore, the combination of both –omics platforms used in the present work offers an opportunity to characterize allergic phenotypes.

2 | MATERIALS AND METHODS

2.1 | Patients

Twenty-five individuals (aged 18–55) were recruited between January 2013 and January 2016. All subjects provided written informed consent. The protocol was approved by the Committees of Research and Ethics from the participant Hospitals. These patients had been deeply studied for oral mucosa remodeling in a previous study.¹⁰ From these, six subjects were nonallergic and used as controls. All patients were interviewed to establish their clinical history. Inclusion criteria for allergic patients were as follows: a positive skin prick test to profilin but negative to lipid transfer proteins commercially available extracts, positive specific IgE to profilin (>0.35 kU/L), but negative to both Pru p 3 and Bet v 1 (ImmuneCAP and ISAC CAP, Thermo Fisher Scientific, Uppsala, Sweden) (Data S1, Table S1). Patients underwent an oral challenge using a pure profilin extract. Clinical status was determined by the clinical history of food allergy and the outcome of the oral challenge. Patients who suffered systemic reactions affecting several organs like urticaria and asthma were phenotyped as “severe profilin-allergic patients” ($n = 6$). Patients who presented local or subjective reactions during oral challenges such as OAS, angioedema or oral pruritus, were considered as “non-severe profilin-allergic patients” ($n = 12$). Individual data are included in the Table S2.



GRAPHICAL ABSTRACT

Altered energy metabolism is connected to the immune cell activation. Sphingolipid metabolism variations and downregulation of platelet-related transcripts in severe allergic patients point to an alteration of platelet functions. Multiple lipid membrane mediators including lysophosphatidylcholines (LPC), lysophosphoserines (LPS), and lysophosphoethanolamines (LPE) were found increased in severe allergic patients.

Allergic patients who had received any pollen extract immunotherapy in the last 5 years, or presented uncontrolled asthma, were excluded.

2.2 | Sample collection and processing

Whole blood was drawn before the profilin challenge and was divided into two tubes: one for plasma extraction and another for PBMCs isolation. Briefly, 20 mL of peripheral blood were collected to obtain plasma by centrifugation and PBMCs using Ficoll-Paque (GE Healthcare™, Chicago, Illinois, USA) density gradient centrifugation. Samples were stored at -80°C until metabolomic and transcriptomic analyses were performed.

2.3 | Metabolomic analyses

Plasma samples were measured using a multiplatform analysis: liquid and gas chromatography coupled to mass spectrometry (LC-MS and GC-MS, respectively). Both techniques followed previously described methodologies.^{21,22} Full descriptions of sample preparation, instrumental description, data treatment, and metabolites identification are available in SI-Part 1. The data are available at Metabolomic Workbench platform (access number ST000980, <http://dx.doi.org/10.21228/M82Q3W>).

2.4 | Statistical analyses

3Multivariate analysis was performed using SIMCA P + 14.0 (Umetrics, Umeå, Sweden). Principal component analysis (PCA), a nonsupervised model, was used to observe data patterns. Likewise, orthogonal and partial least-square discriminant analysis models (OPLS-DA and PLS-DA, respectively), which are supervised models, were used to test real

differences between the case groups. For LC-MS data, logarithmic transformation and center scaling were used, whereas for GC-MS, unit variance scaling was applied to all variables. Robustness of the models was evaluated based on R^2 (explained variance) and Q^2 (capability of prediction) scores.^{23,24} Regarding OPLS-DA, the variability between groups was explained only by the separation in the x-axis, while the y-axis was not related (or orthogonal). This was validated by the cross-validation tool²⁴ and by the “leaving-1/3-out” approach.²⁵ Differences among the experimental groups were tested using the nonparametric Kruskal-Wallis test with a Benjamini-Hochberg (BH) correction to identify potential biomarkers. Additionally, significant differences between “mild vs severe” and “moderate vs severe” — obtained using Mann-Whitney U test with a BH p -value correction — were added, to improve the biological interpretation. Statistical significance was set at 95% level ($P < 0.05$). Statistical analyses were performed using Matlab R2015a (Mathworks, Natick, Massachusetts, USA) software. The MetaboAnalyst (v 4.0) online tool was used for heatmaps and hierarchical clustering.

2.5 | Transcriptomic analysis

2.5.1 | RNA extraction: quantitation and integrity check

RNA was extracted from Ficoll®-isolated PBMCs using RNeasy® Mini Kit (Qiagen, Hilden, Germany) with DNase treatment following manufacturer procedure. RNA concentration was determined using a NanoDrop™ 2000/2000c Spectrophotometer, and its quality was assessed with Experion RNA StdSens analysis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), establishing RQI as an indicator of quality. Following manufacturer recommendations, those samples with an $\text{RQI} \geq 7$ were

selected for the microarray experiments. Samples p2 and p3 from the “nonallergic group” and p24 from the “severe group” did not satisfy quality requirements and were excluded from transcriptomic analysis.

2.5.2 | Microarray target preparation and hybridization

The gene expression profile of the samples was analyzed using GeneChip Human Gene 2.1 ST strips (Affymetrix, Thermo Fisher Scientific Santa Clara, California, USA). Following manufacturer instructions, 100 ng of RNA from each sample was hybridized using GeneChip™ WT PLUS Reagent Kit. Details of hybridization are found in SI-Part 2.

2.5.3 | Analysis of results

CEL raw files were normalized and transformed into expression measures using Expression Console Software (Affymetrix). The Robust Multi-array Average (RMA) algorithm was used for background correction, normalization, and summarization of the probe-set level expression of the samples.

Gene-level expression analysis was performed using the refined metabolomic classification, with Transcriptome Analysis Console (Affymetrix). A gene was considered differentially expressed if it had a fold change (FC) higher than 2 or lower than -2 with an ANOVA p -value < 0.05 .

The pathways involved were acquired by ingenuity pathway analysis (IPA, Qiagen). Gene Set Enrichment Analysis (GSEA) of the RMA normalized samples was performed using the following parameters: number of permutations = 100, collapse dataset to gene symbols = true, and permutation type = phenotype. Raw data can be found on the GEO database (access number GSE114707).

2.5.4 | qRT-PCR analysis

Twenty genes related to the main significant pathways found with GSEA and IPA (inflammatory response, platelet functions, fatty acid metabolism, and histone modification) were selected for validation studies by quantitative RT-PCR (qRT-PCR, Table S3).

High Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, California, USA) was used for the validation according to the manufacturer instructions; briefly, 1 μ g of total RNA was reverse transcribed in a final volume of 20 μ L. Primers were designed using OligoArchitect™ (Sigma-Aldrich, San Luis, Missouri, USA), and qRT-PCR was performed using SYBR Green master mix (Takara Kusatsu, Japan) in the equipment Real Time HT 7900 (Applied Biosystems). Reactions were run in triplicate. Expression data were normalized to the median of housekeeping gene *B2M*, and the results were analyzed using the $2^{-\Delta\Delta CT}$ method.²⁶ Details are found in SI-Part 2.

3 | RESULTS

3.1 | Metabolic profiling and patient classification

Metabolomic profiles of plasma samples were obtained using LC-MS and GC-MS. Initially, from 615 and 506 chemical signals obtained by LC-MS

in positive and negative ionization modes, respectively, 349 and 400 complied with the quality criteria. In addition, 95 metabolites per sample were obtained using GC-MS. Data quality was assessed by clustering quality control (QC) measurements in a nonsupervised model using PCA for the three techniques (Figure S1). Trends of the samples were examined; as an example, the PCA model from LC-MS data in positive mode showed no clear clustering of patients according to their clinical status (Figure 1A). However, individuals with a clear clinical status—“nonallergic” and “severe profilin-allergic patients”—were modeled using a supervised model. Therefore, in order to obtain the differences exclusively according to the class,²⁷ an OPLS-DA model was performed (Figure 1B). This model showed good quality parameters ($R^2 = 0.99$, $Q^2 = 0.75$) and was validated using the “1/3-out” approach, showing a prediction accuracy of 92% and was used to assign a class to “Non-severe profilin-allergic patients” (Figure 1B), following a similar approach to other authors.²⁸⁻³⁰ Samples were grouped within circles according to the OPLS-DA mathematical algorithm (Table S4). Patients with a predicted score higher than 0.75, related to nonallergic, were classified as “mild.” Those with a score higher than 0.75, closer to “severe profilin-allergic patients,” were classified as “severe,” whereas the rest of the samples were classified as “moderate.” Consequently, four groups were stratified: “nonallergic,” “mild,” “moderate,” and “severe.” To sum up, the model allowed the classification of “Non-severe profilin-allergic patients” that could not be properly classified in the oral challenge setup.

Samples were plotted according to their metabolomic classification using a PCA model, which showed a clear tendency to separate the “mild” group from the “severe” and “moderate” groups (Figure 1C). Furthermore, aiming to show the differences between groups, discriminant analysis using PLS-DA was performed for each pair of groups (Figure S2). The results produced at least one model for each pairwise comparison with high-quality parameters. These results demonstrated that there are metabolic differences between the groups.

3.2 | Metabolic description between allergic phenotypes

After multivariate analysis, significant variables were selected using univariate analysis. The numbers of significant variables between groups were summarized using Venn diagrams. More differences were observed between “mild vs severe” than between “severe vs moderate” phenotypes (Figure 2A). Moreover, FC was represented by heatmaps using hierarchical clustering within the allergic groups (Figure S3). Overall, results suggest that there are specific features which can characterize each phenotype, especially the “severe.”

Once significant features in LC-MS were found, identification through MS/MS fragmentation was performed. Together with GC-MS metabolites, a total of 74 compounds were listed (Table S5), encompassing carbohydrates, sphingolipids, lysophospholipids, carnitines, amino acids, fatty acids, and organic acids. Their FCs were calculated in the pairwise comparisons and summarized in Table S6. Most of the metabolites presented increased levels in “severe” compared to “mild” group ($n = 49$).

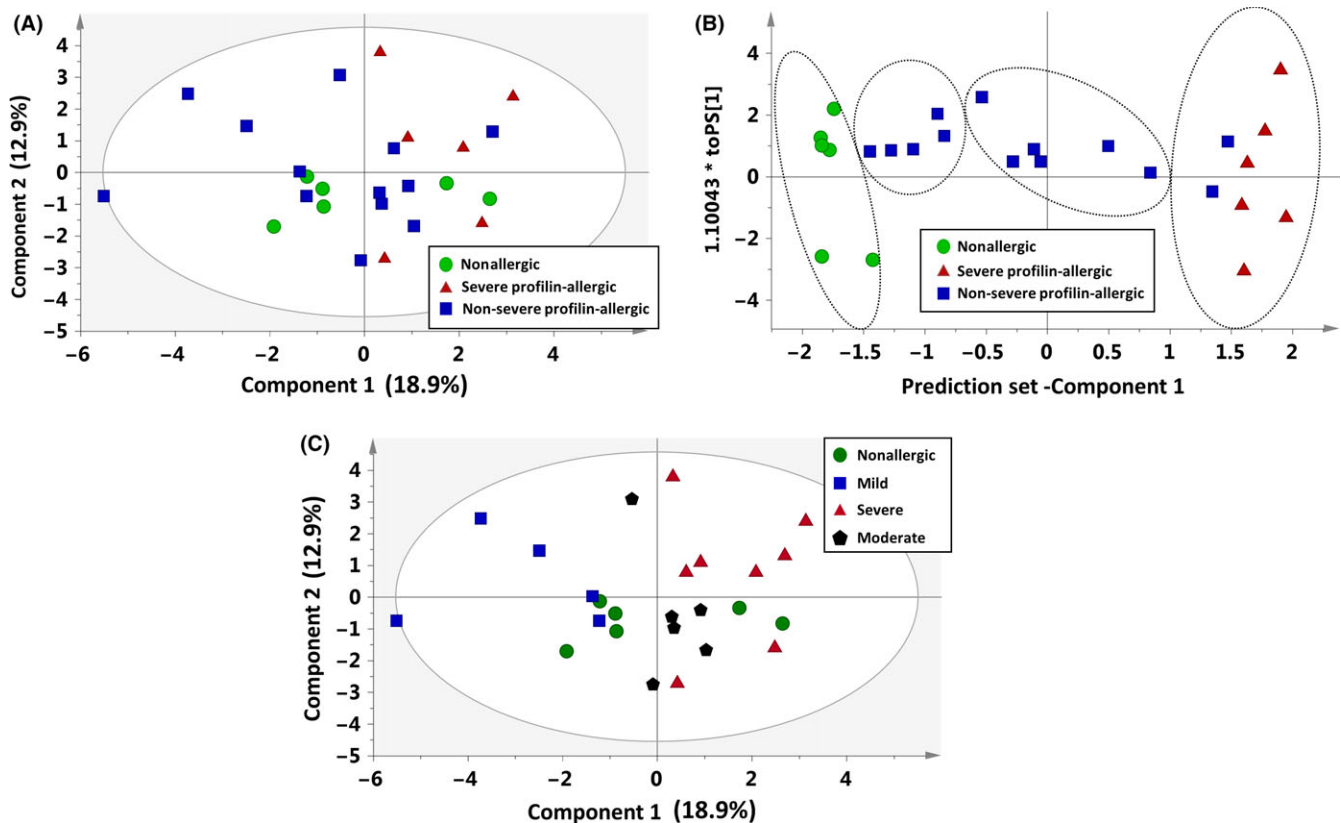


FIGURE 1 Unsupervised and supervised multivariate analysis of LC-MS-positive mode data used for refined classification of “Non-severe profilin-allergic patients.” (A) PCA model of the sample set. Key: “nonallergic” (●; green circle), “Non-severe profilin-allergic patients” (■; blue square), “severe profilin-allergic patients” (▲; red triangle). (B) OPLS-DA prediction model; $R^2 = 0.99$, $Q^2 = 0.75$. Key: workset samples (green circle ● – “nonallergic” subjects; red triangle ▲ – “Severe profilin-allergic patients”), prediction set samples (blue square ■ – “Non-severe profilin-allergic patients”). (C) PCA model plotted using new classification. Key: “nonallergic” (●; green circle), “mild” (■; blue square), “moderate” (◆; black pentagon), “severe” (▲; red triangle). Log transformation and center scaling were used for the models.

The general observed changes in the “severe group” were as follows: decreased levels of hexoses, pentoses, pyruvate, and sphinganine-C17; an increase along severity in the levels of, most lysophosphocholines (LPCs), sphingosine-1-phosphate (S1P), sphinganine-1-phosphate, and lactate. Furthermore, fatty acids such as myristic and lauric acid were diminished in “mild” and “moderate,” and increased in the “severe” group. Trajectories of these metabolites are shown in Figure 2B.

3.3 | Gene expression analysis

From the 48 226 transcripts analyzed, 596 transcripts were identified as significantly differentially expressed between the experimental groups (Figure 3A). These differences were depicted in the Venn diagrams. Major differences were observed between allergic phenotypes, especially in the “severe vs mild” comparison, with 220 differentially expressed transcripts (Figure 3A), and between “severe vs moderate,” with 87 (Figure 3B). Validation of the transcriptomic analysis was assessed by qRT-PCR, where 95% of the selected transcripts showed the same expression pattern as in the microarray experiments. Thus, the results obtained can be considered reliable (Figure S4).

Afterward, we selected the 100 most differentially expressed genes to depict a heatmap (Table S7). Transcripts were classified into

four clusters according to their expression pattern (Figure 3C). Cluster 1—upregulated in the “severe” phenotype and downregulated in “mild”—contained essentially H/ACA box snoRNAs (SNORA). Cluster 2—which was downregulated in the “mild” group—included a heterogeneous group of genes. Cluster 3—decreased in severe patients with respect to the other groups and thus constituted the best biomarkers for the “severe” phenotype—includes genes closely related to coagulation and histone modifications. Finally, cluster 4—where genes were downregulated in “severe” compared to the “mild” group—contained small nucleolar RNAs (snRNA), their variants, and genes related to protein biosynthesis and growth factors.

3.4 | “Severe” patients display a differential transcriptomic profile

We performed pathway analysis aiming to match the characteristic genes of the “severe” profile with associated biological processes. Two types of complementary analyses were carried out. First, we compared separately “severe vs mild” (Table S8-A) and “severe vs moderate” (Table S8-B) using GSEA. Then, we repeated the analysis of the previous comparisons with IPA (Table 1). GSEA results indicated that the pathways of DNA repair, inflammatory response, oxidative

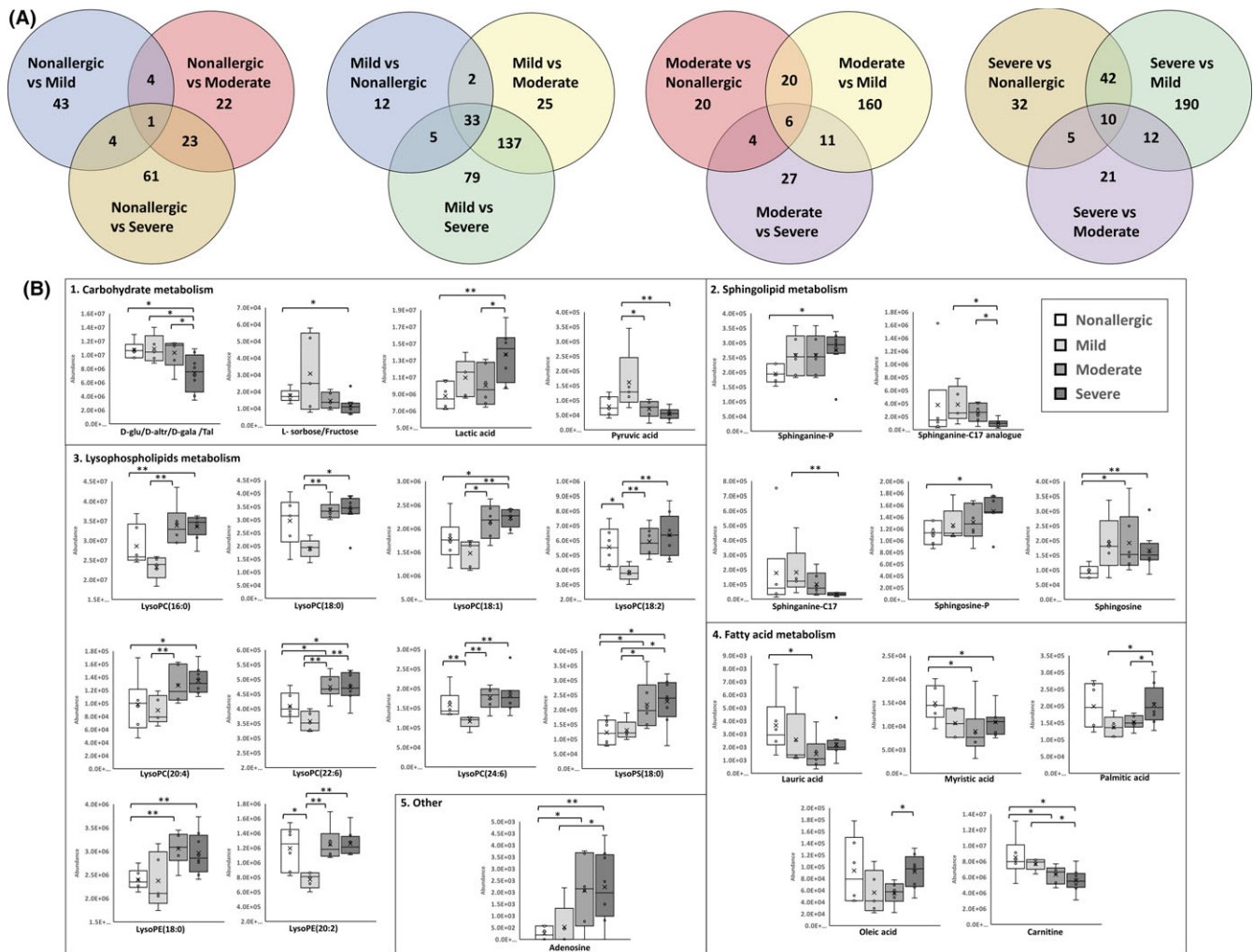


FIGURE 2 Statistical significant differences between the groups within the three techniques. (A) Venn diagrams representing the differences in each group against the rest: “nonallergic vs mild” (blue), “nonallergic vs moderate” (red), “nonallergic vs severe” (brown), “mild vs moderate” (yellow), and “moderate vs severe” (purple). (B) Trajectories of representative metabolites classified according to their biochemical groups: 1. Carbohydrate metabolism, 2. Sphingolipid metabolism, 3. Lysophospholipids metabolism, 4. Fatty acid metabolism, 5. Other (Key: “nonallergic”: white square, “mild”: light gray square, “moderate”: gray square, “severe”: dark gray square). The central box covers the interquartile range with the median indicated by the line within the box and the mean of each experimental group is represented by “X.” The whiskers extend to the minimum and maximum values, and outlier values are shown individually. *Mann-Whitney *U* test *p*-value < 0.01. *Mann-Whitney *U* test *p*-value < 0.05

phosphorylation, fatty acid metabolism, the complement system, and coagulation were negatively correlated with the “severe” phenotype. Likewise, IPA obtained pathways related to endothelial cells, platelet phagocytosis, and platelet functions—such as activation and, binding and adhesion, among others. Additionally, the activation state (Activation Z-score) of the significant pathways, which can be positive—pointing to pathway activation—or negative—for inactivation—was measured. Most of these functions had a negative value for “severe” patients (Table 1).

These results suggest that coagulation- and platelet-related pathways are downregulated in “severe” patients and might be their specific transcriptomic profile. Moreover, pathways and transcripts related to fatty acid metabolism and histone modification were increased in “mild” and “moderate” groups while decreased in “severe” phenotype.

3.5 | Severe allergic phenotypes present altered platelet functions

Taking into account these findings, we decided to focus on coagulation- and platelet-related transcripts. Specifically, those transcripts observed as statistically significant for IPA software, and genes related to platelet functions were analyzed (Figure 3C).

The trajectories among the groups were plotted to corroborate that the selected genes had a different expression pattern in the “severe” phenotype (Figure 4). The plots showed a significant decrease in the expression of these genes in the “severe” group compared to the rest. Among them there were genes related to platelet adhesion (*GP1BA*, *GP9*, *GP6*, and *SELP*); activation (*P2RY12*, *ALOX12*, and *PTGS2*); both shape change and granule secretion (*MYL9* and *SDPR*)³¹;

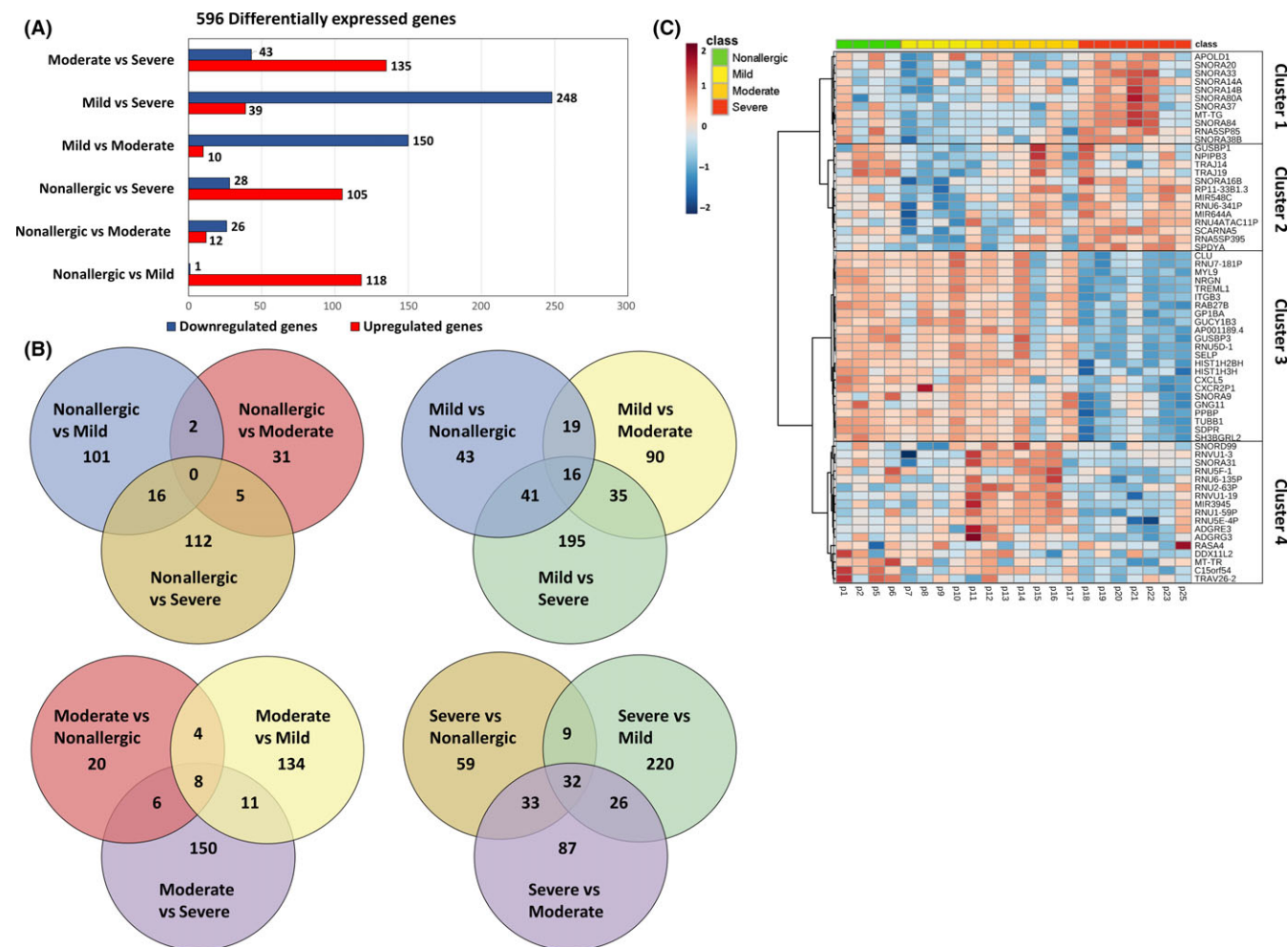


FIGURE 3 Statistically significant transcript differences between experimental groups. (A) Genes differentially expressed among the phenotypes. Red and blue bars represent, respectively, genes upregulated and downregulated in the first group of the comparison. (B) Venn diagrams representing the differences in each group against the rest: “nonallergic vs mild” (blue), “nonallergic vs moderate” (red), “nonallergic vs severe” (brown), “mild vs moderate” (yellow) and “moderate vs severe” (purple). (C) Heatmap of the 100 top differentially expressed genes. Upregulated genes are represented in red and downregulated are shown in blue. ANOVA p -value < 0.05.

aggregation (*ITGB3*, *ITGA2B*, and *TREML1*)³²; as well as other genes related to platelet functions (*PPBP*, *CLU*, and *NRGN*).^{33,34} These transcripts and their platelet-associated functions were represented in Figure 5 to have a better understanding of platelet dysfunctions in the “severe” phenotype.

Regarding metabolomics, we also observed some metabolic changes related to platelet activation: S1P and LPs were incremented in the “severe” group compared to the intermediate allergic phenotypes (“mild” and “moderate”), while sphingosine was decreased (Figure 2B). An integrated view of both metabolomic and transcriptomic changes supporting altered platelet functions in “severe” allergic individuals is represented in Figure 5.

4 | DISCUSSION

Finding suitable clinical models of allergy disease progression is not easy. In particular, identifying homogeneous severe allergic

phenotypes, which are critical to understand allergy evolution, is an arduous task. We took advantage of the unique model that these grass pollen-allergic patients represent—those resident in high pollen exposure areas who develop severe food allergic profilin-mediated reactions. These reactions identify a patient group with a severe allergic phenotype that apparently loses tolerance to the allergen.

The aim of this work was both to identify potential biomarkers and to understand underlying causes for severity progression. We used a combined approach of two -omics, metabolomics and transcriptomics, and generated a model for patient classification.

Previous studies have shown that the use of mathematical algorithms based on objective parameters—such as transcriptome profiles—is useful to improve the clinical classification of patients.^{28–30} In this work, we have established a classification based on the metabolic profile of the patients. First, we included nonallergic subjects and identified severe allergic phenotypes by their clinical history and their response to the oral challenge test with pure profilin. Using these two extreme phenotypes, we obtained a

TABLE 1 Ingenuity pathway analysis (IPA) significant results for “severe vs mild” and “severe vs moderate” comparisons, and the functions were ranked by *p*-value

Categories	Diseases or functions annotation	<i>p</i> -value	Activation z-score ^a	<i>n</i> ^b
A. Severe vs mild				
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response, organismal functions	Activation of blood platelets	1.01E-05	−1.091	5
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response	Binding of blood platelets	1.07E-04	−1.019	4
Cell-to-cell signaling and interaction	Binding of blood cells	1.90E-04	−0.605	8
Cardiovascular system development and function, cell-to-cell signaling and interaction	Binding of endothelial cells	2.15E-04	−1.095	6
Cell-to-cell signaling and interaction, cellular function and maintenance, hematological system development and function, inflammatory response	Phagocytosis of blood platelets	2.55E-04	—	2
Cell-to-cell signaling and interaction, cellular function and maintenance, inflammatory response	Phagocytosis of blood cells	2.58E-04	−1.103	4
Cellular movement	Chemotaxis	3.58E-04	−0.919	9
Cellular movement, immune cell trafficking	Leukocyte migration	3.76E-04	−0.505	10
Cardiovascular system development and function, cell-to-cell signaling and interaction	Adhesion of endothelial cells	3.85E-04	−1.079	5
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response	Adhesion of blood platelets	4.52E-04	—	3
Cell-to-cell signaling and interaction	Aggregation of cells	9.58E-04	−0.556	6
Cell-to-cell signaling and interaction, hematological system development and function	Aggregation of blood cells	9.83E-04	−0.783	5
Cell-to-cell signaling and interaction, hematological system development and function	Activation of blood cells	1.22E-03	−2.021	9
Cardiovascular system development and function, organismal development	Angiogenesis	1.43E-03	−1.294	10
Cell morphology, hematological system development and function	Conversion of blood cells	1.51E-03	—	2
Cardiovascular system development and function, cellular movement	Cell movement of endothelial cells	1.63E-03	−1.404	7
Tissue development	Accumulation of cells	1.70E-03	0.243	4
Carbohydrate metabolism	Metabolism of glycogen	1.74E-03	—	2
Cardiovascular system development and function, cellular movement	Movement of vascular endothelial cells	2.01E-03	−0.849	5
Cell-to-cell signaling and interaction, cellular movement, hematological system development and function, immune cell trafficking	Recruitment of leukocytes	2.45E-03	—	4
B. Severe vs moderate				
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response	Binding of blood platelets	3.19E-12	−2.369	9
Cell-to-cell signaling and interaction	Binding of blood cells	6.75E-11	−3.219	15
Cell-to-cell signaling and interaction	Adhesion of blood cells	1.44E-10	−2.932	14
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response	Adhesion of blood platelets	1.52E-10	−1.632	7
Cell-to-cell signaling and interaction, hematological system development and function	Aggregation of blood cells	3.20E-10	−2.521	11
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response	Aggregation of blood platelets	5.19E-10	−2.298	10
Hematological system development and function	Hemostasis	4.38E-09	−2.107	10
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response, organismal functions	Activation of blood platelets	1.75E-08	−1.318	7
Cardiovascular system development and function, cell-to-cell signaling and interaction	Binding of vascular endothelial cells	2.29E-06	−2.620	7

(Continues)

TABLE 1 (Continued)

Categories	Diseases or functions annotation	p-value	Activation z-score ^a	n ^b
Cardiovascular system development and function, cell-to-cell signaling and interaction	Binding of endothelial cells	2.68E-06	-2.170	8
Cardiovascular system development and function, cell-to-cell signaling and interaction	Adhesion of endothelial cells	3.31E-06	-1.563	7
Cellular movement, hematological system development and function, immune cell trafficking, inflammatory response	Infiltration by neutrophils	3.39E-06	-2.159	5
Cardiovascular system development and function, cellular movement	Chemotaxis of vascular endothelial cells	3.67E-06	-1.934	4
Cellular movement, hematological system development and function, immune cell trafficking	Cell rolling of leukocytes	7.83E-06	—	4
Cellular movement	Arrest in cell movement of myeloid cells	8.86E-06	—	3
Cellular movement, hematological system development and function, immune cell trafficking, inflammatory response	Cell rolling of phagocytes	8.86E-06	—	3
Cellular movement, hematological system development and function, immune cell trafficking, inflammatory response	Cell movement of neutrophils	8.91E-06	-2.541	7
Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking, inflammatory response	Adhesion of neutrophils	9.21E-06	-2.177	5
Immunological disease	Allergy	1.05E-05	—	10
Cell-to-cell signaling and interaction, hematological system development and function	Activation of blood cells	1.045E-05	-2.620	12
Cellular assembly and organization	Binding of cytoskeleton	1.62E-05	—	3

The source was IPA for all categories.

^aStatistical value based on the match between expected relationship and observed gene expression that predicts the activation state of pathways (positive activation; negative inactivation).

^bNumber of significant genes present in the pathway.

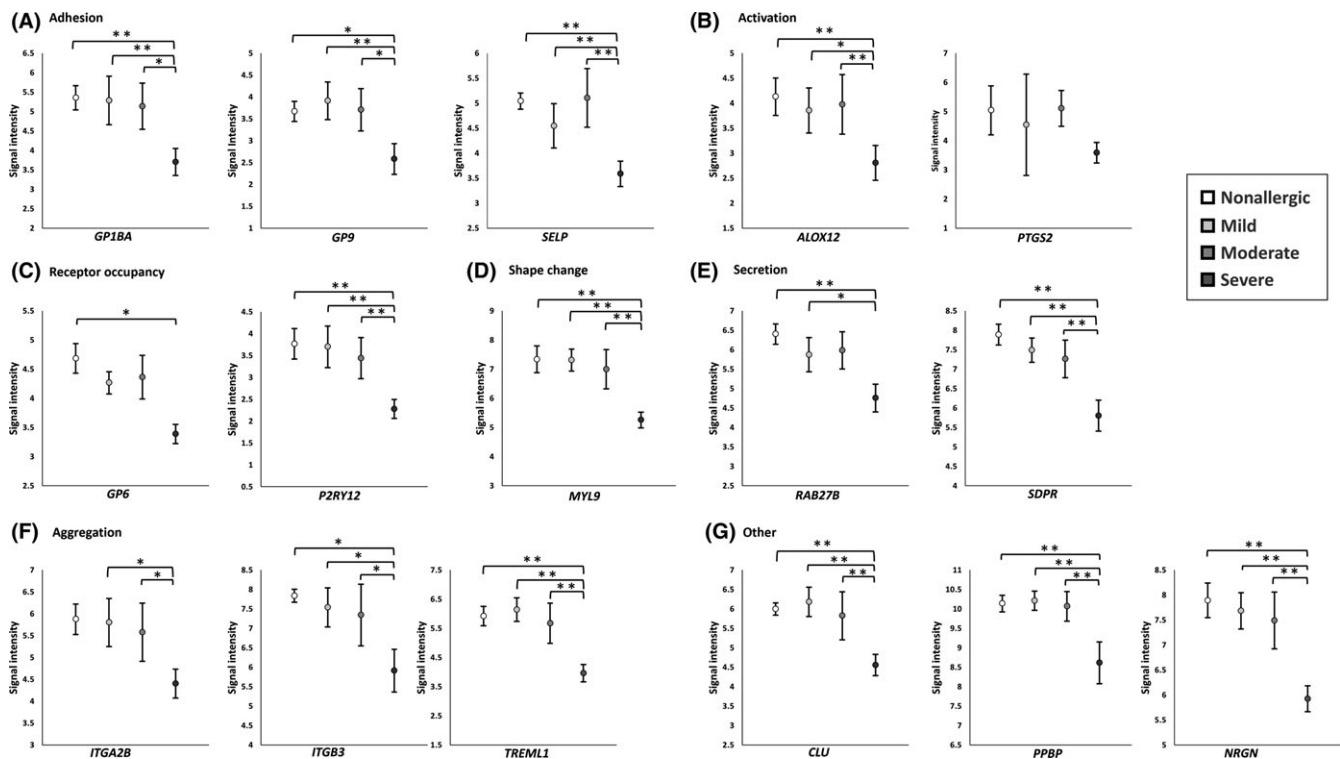


FIGURE 4 Trajectories of significant transcripts associated with platelet functions. (A) Adhesion, (B) Activation, (C) Receptor Occupancy, (D) Shape Change, (E) Secretion, (F) Aggregation, (G) Other (Key: “nonallergic”: white square, “mild”: light gray square, “moderate”: gray square, “severe”: dark gray square), 95% confidence interval limits for the mean (central circle) of each experimental group are indicated ** ANOVA p-value < 0.001. *ANOVA p-value < 0.05.

mathematical algorithm that was used to refine the clinical classification of the “Non-severe profilin-allergic patients.” It is important to point out that these phenotypes cannot be perfectly classified upon oral provocation, as many times the provocation procedure must be stopped due to intense local reactions.³⁵ This new classification represents a potential tool for patient stratification that should be validated in further studies and is in line with current approaches to understand disease phenotypes.^{28–30} This allowed us to analyze the evolution of allergic inflammation along severity. Interestingly and unexpectedly, all patients that were clinically classified as “severe” shared common metabolomic and transcriptomic profiles, thus confirming the correlation between clinical and –omics classification.

Afterwards, we performed data analysis of both –omics to find out specific signatures, to identify biomarker candidates and biological pathways associated with the acquisition of a severe phenotype. The different metabolite levels and transcript expression kinetics provided a global view of the biological processes associated with inflammation evolution.

The first set of downregulated transcripts in “severe” patients consisted of DDX11L2 and MT-TR, which are involved in translation

initiation, nuclear and mitochondrial splicing, and ribosome-spliceosome assembly.³⁶ In parallel, we observed an increase in urea, suggesting an increase in protein catabolism.

Marked changes in cell energy metabolism were also found. Hexoses, pentoses, and pyruvate are consumed and metabolized into lactate in the presence of oxygen. In agreement with our results, existing –omics-based studies have reported that immune cells change their metabolism to cope with the increasing demand resulting from the need to synthesize biological precursors. This is known as “aerobic glycolysis” or “Warburg metabolism”^{37,38} and has been described in tumoral cells, asthma, and inflammation.^{39–41} Furthermore, with GSEA, we also identified different biological processes reduced in “severe” patients: such as oxidative phosphorylation, fatty acid metabolism, and adipogenesis (Table S8); all supporting the switch to Warburg metabolism.

Analyzing lipid metabolism, we observed a progressive increase in arachidonic acid (ARA) precursors—palmitic and oleic acids—among allergic phenotypes. Meanwhile, shorter fatty acids—myristic and lauric acids—were decreased in the intermediate phenotypes (“mild” and “moderate”) but increased in the “severe” group. Furthermore, carnitines levels followed a reduction trend along severity.

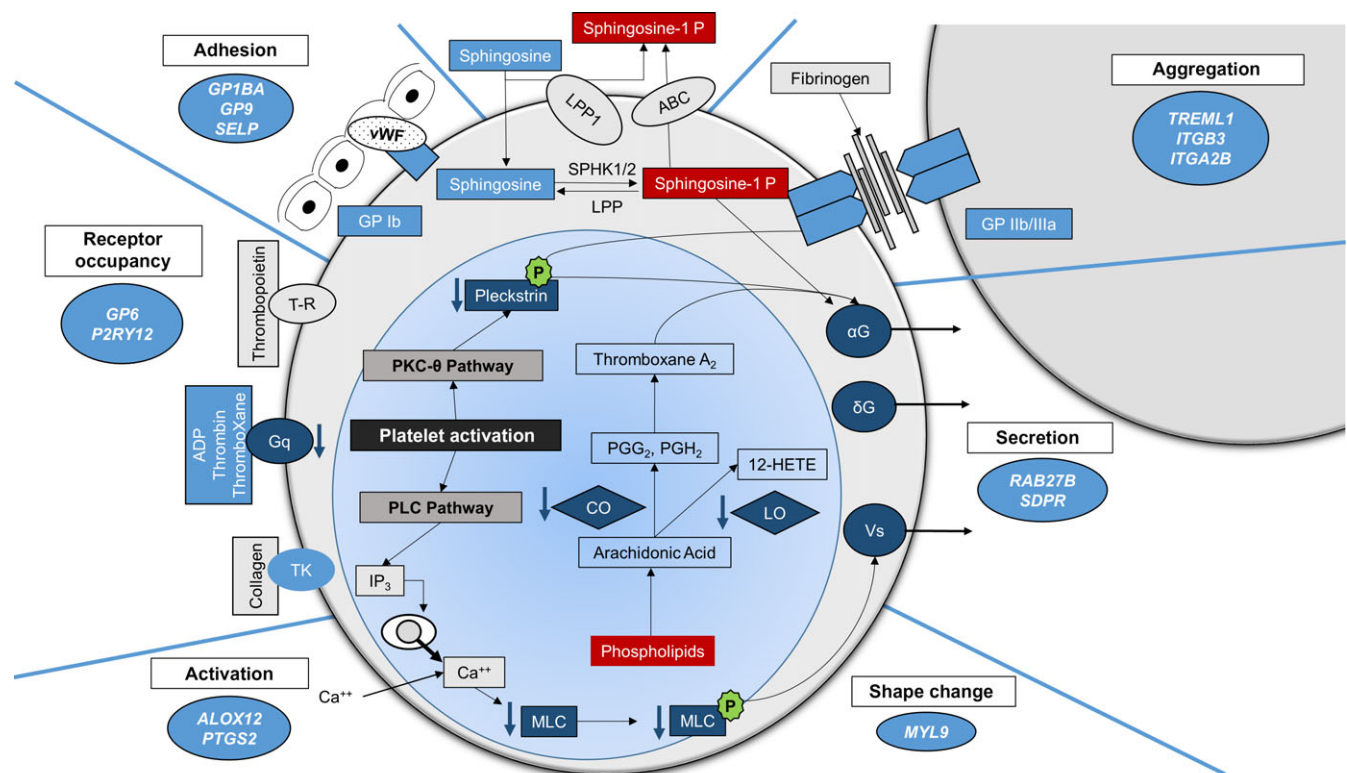


FIGURE 5 Schematic representation of the proposed platelet dysfunction mechanism in “severe” phenotype. Molecules and functions represented in blue are diminished: Decreased metabolites and downregulated transcripts are represented in light blue. Molecules indirectly associated with transcripts are represented in dark blue and marked with a down arrow. Increased metabolites are marked in red. All functions are divided by blue lines and their associated downregulated transcripts are shown in a blue ellipse. Platelet activation pathways are included in a light blue circle. 12-HETE, 12-hydroxyeicosatetraenoic acid; ABC, ABC transporter; CO, cyclooxygenase; GP, glycoprotein; Gq, G protein receptor; IP₃, inositol triphosphate; LO, lipoxygenase; LPP, lipid phosphate phosphatase; MLC, myosin light chain protein; PGG₂, prostaglandin G₂; PGH₂, prostaglandin H₂; PKC-θ, protein kinase C θ; PLC, phospholipase C; SPHK1/2, sphingosine kinase 1/2; TK, tyrosine kinase receptor; T-R, thrombopoietin receptor; Vs, vesicles; vWF, von Willebrand factor; αG, alpha granules; δG, dense granules.

In contrast, we did not see changes in the levels of ARA between groups, matching previous reports in which exposure to allergens did not change the levels of this fatty acid in plasma.²⁰ One possible explanation for these results is that, instead consuming the fatty acids by beta-oxidation, they are likely being used for the synthesis of inflammatory mediators.^{42,43} Perhaps, the most interesting metabolic signature is related to sphingolipids. We detected a progressive increase in S1P—a key mediator that exerts a multitude of effects in different tissues—with severity. S1P is capable of regulating gene transcription by inhibiting histone deacetylases,⁴⁴ which concurs with the downregulation of histone modification-related genes (*HIST1H2BH* and *HIST1H3H*). These had a negative association with DNA repair in the “severe” phenotype,⁴⁵ suggesting modifications in epigenetic regulation networks.

Interestingly, we have identified a set of downregulated transcripts involved in platelet functions: formation of adhesion complexes (*GP1BA* and *GP9*, *SELP*); receptors (*GP6*, *P2RY12*)⁴⁶; aggregation of complexes (*ITGB3*, *ITGA2B*)⁴⁷; synthesis of HETE-12 and Thromboxane A2 (*ALOX12*, *PTGS2*)⁴⁸; and both shape change induction and granule secretion processes (*MYL9*, *SDPR*, *RAB27B*).^{49,50} Moreover, in the GSEA and IPA analyses, several pathways related to platelets and coagulation were significantly downregulated in the “severe” group. Aligned with these findings, we have observed different metabolic changes. We detected a decrease in sphingosine and xenosphingolipids (sphinganine-C17) in the “severe” group. Sphingosine is a precursor of S1P, and their decreased levels along severity suggest a continuous biosynthesis of S1P. Furthermore, in a recent study, an inverse association of intestinal microbial-derived sphingolipids with childhood food allergy was established, highlighting the importance of those metabolites in the allergic disease.⁵¹ In addition, different studies have demonstrated that S1P has a role in inflammation and that activated platelets are able to produce, store, and release it by an ABC transporter.^{52–55} Besides, S1P can be degraded by the LPP1 platelet outer surface receptor⁵⁶ (Figure 5). Finally, we observed an increase along the severity of lysophospholipids (LPCs, LPS, and LPEs), as other authors have previously described in asthma and other inflammatory pathologies.^{17,20,57–59} These lysophospholipids and ARA precursors, increased in “severe” patients, are involved in platelet activation in inflammatory environments.⁶⁰ Recent evidence revealed that the lung is a site of platelet biogenesis and a reservoir for hematopoietic progenitors.⁶¹ This creates a whole range of new possibilities for understanding the role of platelets in airway inflammation. Our findings point to platelet dysfunction in the “severe” phenotype (Figure 5).

These findings open up the possibility for considering new intervention strategies oriented to the stabilization of inflammation/repair systems, *for example*, using supplement-based interventions to replenish metabolic precursors that are highly consumed, as other studies have pointed out for asthma and allergy.^{19,62–64} Altered energy metabolism could also constitute a pharmacological target. Understanding in detail the role of platelets could become a completely different approach to control severe allergic phenotypes.

The combined analysis of transcripts and metabolic alterations in allergic patients that have been stratified based on their severity sheds

some light on the progression of this pathology. Interestingly, the role of platelets in inflammation and related pathologies, such as cancer and autoimmune diseases, has been already suggested.^{65–70} We describe new mechanisms, pointing to platelet dysfunction, reduced protein synthesis, and a switch to Warburg metabolism, that are associated with severe allergic inflammation. Further studies in other severe allergy models, together with prognosis strategies based on the omics approaches, will allow for a better comprehension of the complex network between platelets and the immune system.

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CONFLICTS OF INTEREST

Domingo Barber has received consultancy fees from ALK and Aimmune therapeutics companies. Tomás Chivato has received lecture fees from URIACH and FAES laboratories. Montserrat Fernández Rivas consulting fees from Aimmune, DBV, Schreiber Foods, and lecture fees from ALK, Allergy Therapeutics and Thermofisher Scientific. The rest of the authors have no conflict of interest. All authors have read and approved the manuscript.

AUTHOR CONTRIBUTIONS

D.B. was the PI and together with C. Barbas, M.M.E., T.Ch., and A.V. designed and supervised the research. D.O. performed the metabolomics analysis and data treatment. L.M.B. performed the transcriptomic analysis and data treatment. J.R.-C., D.R., P.F., and I.M.A. contributed to the metabolomics and transcriptomics biological interpretation. M.S., G.M., M.F.R., T.R., C. Blanco, M.I.A., and C.D. included all study patients. S.A. supervised statistical analysis. 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 at the end of the article.

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SUPPLEMENTARY INFORMATION

Multi-omics analysis Points to Altered Platelet Functions in Severe Food-Associated Respiratory Allergy

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1. PART 1. Detailed description of sample preparation, instrumental description and data pre-treatment for metabolomics analyses.

Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

Sample treatment

For LC-MS, plasma proteins were removed adding 300 μ L of cold (-20 °C) methanol:ethanol (1:1) to 100 μ L 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 $\times$ g for 20 min at 4 °C), and put into a LC vial for analysis. Samples were measured using an Agilent HPLC system (1200 series), coupled with quadrupole-time of flight analyzer (Q-TOF/MS 6520) system (Agilent Technologies, Waldbronn, Germany).

QC Sample Preparation and randomization

Quality control (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 4 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-Quadrupole Time of Flight-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), LC-MS (6520) system (Agilent Technologies, Waldbronn, Germany), as previously described ([1](#)).

Ten μ L of sample were injected into a Discovery HS C18 column (2.1 mm $\times$ 150 mm, 3.0 μ m; Supelco, Sigma Aldrich, Germany), with a guard column Discovery[®] HS C18 (2 cm $\times$ 2.1 mm, 3 μ m; 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. Flow rate was 0.6 mL/min, and 10 μ L of samples were injected. The electrospray source ionization (ESI) data were acquired in both positive and negative ion modes respectively. The capillary voltage was set at 3,500 for ESI (+) and 4,000V for ESI (–). 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. 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 mode. The reference m/z ions were purine (121.0508) and HP-0921 (922.0097) for ESI (+), whereas 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 adopted from previous work (1).

Metabolite Identification

Compound identification for LC-MS was carried out for statistically significant features in three steps: 1) putative identification, 2) LC-MS/MS fragmentation and 3) by commercial standard confirmation. Putative identification was performed in all the 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). ID was confirmed through LC-MS/MS experiments by using a QTOF (model 6520, Agilent). Experiments were repeated in same identical chromatographic conditions as the primary analyses. Ions were targeted using the narrow m/z window (1.3 Da) and 20 V of energy for fragmentation on the quadrupole at the RT previously determined. Comparison of the structure proposed against the obtained fragments led to the confirmation of the identity. Finally, those compounds with an available commercial standard were confirmed by comparison of retention time (RT) and fragmentation pattern distribution. Tentative identification using web databases was carried out on 182 features for LC-MS positive and 194 features for negative polarity. From these, a total of 319 m/z values for both polarities were found and selected for MS/MS experiments. Afterwards, in total 62 m/z values were identified by their fragmentation pattern. Subsequently, 45 metabolites were described with a unique identification for both polarities. Finally, 22 metabolites were confirmed using commercial standards.

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.06.00, 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 615 chemical signals for LC-MS positive ionization mode and 506 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%.

Gas Chromatography coupled to Mass Spectrometry (GC-MS)

Sample treatment

For protein precipitation, 120 μ L of cold acetonitrile (ACN) were added to 40 μ L of each plasma sample in an Eppendorf and were stored on ice for 5 minutes. Then, metabolites were separated by centrifugation (16,000 $\times$ g, 15 min, 4 $^{\circ}$ C). From the resulting supernatant, 100 μ L were transferred to GC vial with insert and were evaporated to dryness (SpeedVac Concentrator, Thermo Fisher Scientific, Waltham, MA, USA). Then, 10 μ L of O-methoxyamine hydrochloride in pyridine (15 mg/mL) were added to each GC vial, and the mixture was vigorously vortex-mixed and ultrasonicated. Methoxymation was carried out in darkness, at room temperature for 16 h. Afterwards, 10 μ L of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were added as catalyst and the solution was further mixed using the vortex. For silylation process, samples were heated in an oven for 1h at 70 $^{\circ}$ C. Finally, 100 μ L of heptane containing 10 ppm of C18:0 methyl ester (IS) were added to each GC vial and vortex-mixed before GC analysis.

GC-Quadrupole-MS equipment

GC-MS analysis was performed by a GC system (Agilent Technologies 7890A) equipped with an autosampler (Agilent 7693) coupled to a mass spectrometer with triple-Axis detector (5975C, Agilent). Two microliters (2 μ L) of the derivatized sample were injected through a GC-Column DB5-MS (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 was applied of 10 min before the next injection. Detector transfer line, filament source and the 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. Several IS injections, a standard mix of fatty acid methyl esters (FAME C8-C30), extraction blanks and 3 QCs samples were injected at the beginning of analysis, following QCs injections every 5 experimental samples and 1 QC injection at the end of worklist. These conditions were adopted 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 version B.07.00 from 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 accuracy improvement 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 (Agilent). Integration of the targets and alignment of the drift were performed on MassHunter Quantitative (Agilent). Raw data filtering was performed by removing those features detected in <50% of all QC samples and with a %RSD >30% in QC samples. Data matrix was normalized using Median Fold Change algorithm.

2. PART 2. Detailed Microarray target preparation and hybridization using GeneChip Human Gene 2.1 ST.

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 cRNA was obtained by *in vitro* transcription (IVT) of the double stranded cDNA. Next, cRNA 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.

Figure 1S. Unsupervised PCA models showing the quality of the data for the three techniques. (Key: QC injections (●; dot), samples (●; grey circle)).

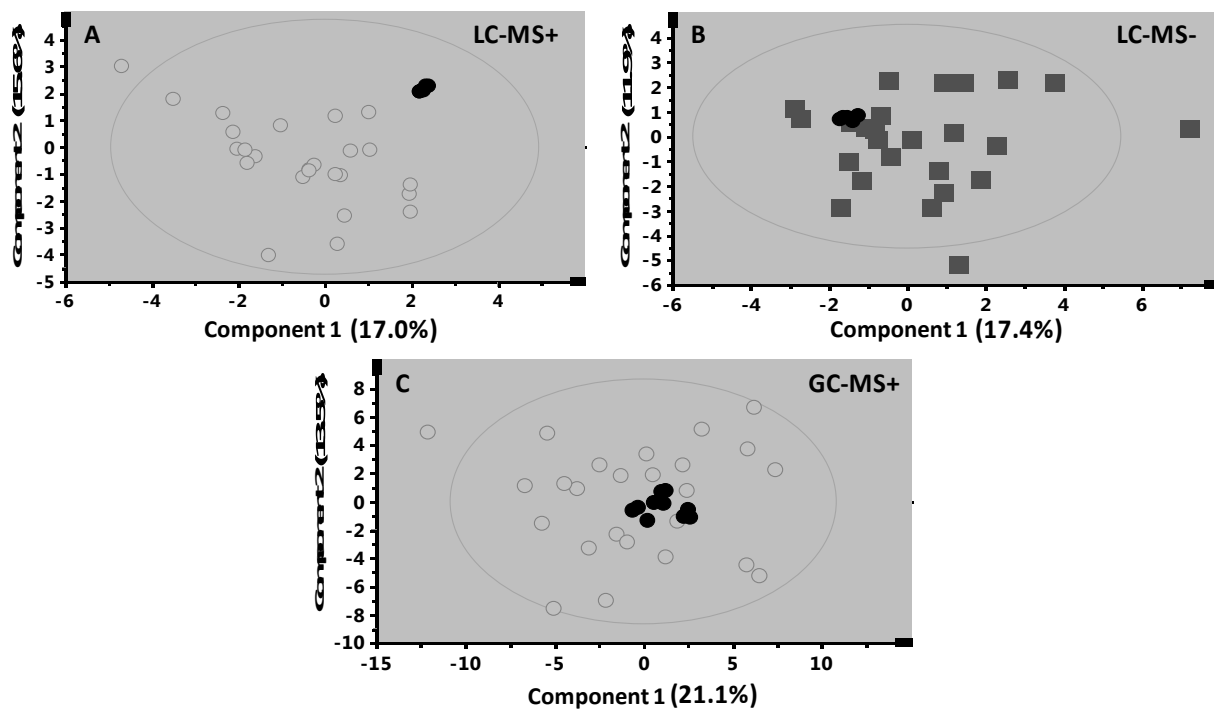


Figure 2S. PLS DA models for each pairwise comparison in the three complementary techniques. (Key: “non-allergic”: circle, “mild”: square, “moderate”: pentagon, “severe”: triangle). Data was Log transform and centre scaled. *Characters in bold pointed out suitable quality parameters ($Q_2 > 0.5$) for the model.

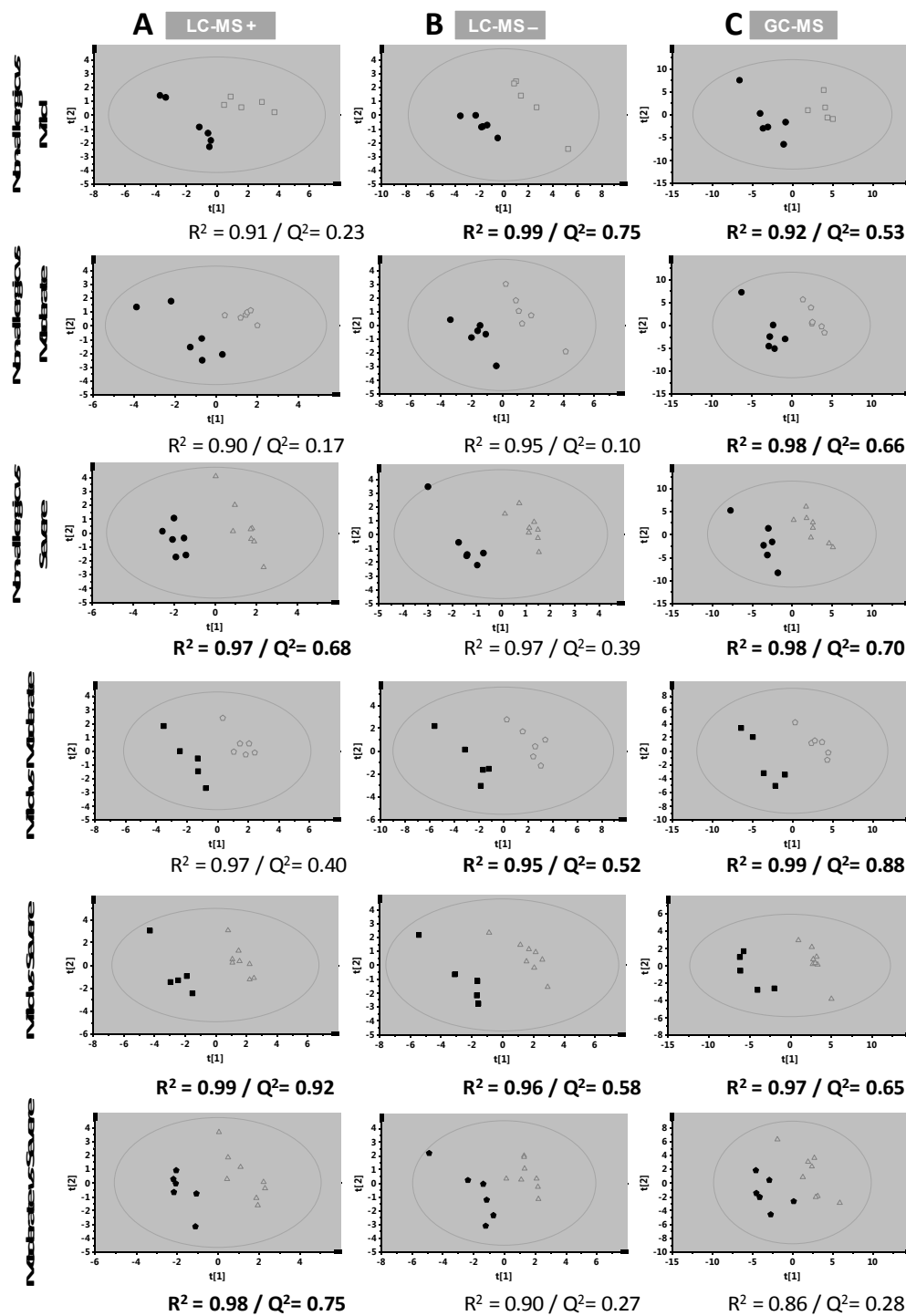


Figure 3S. Significant signals between “mild vs severe” and “moderate vs severe” groups were depicted on heatmaps using hierarchical clustering of the samples. Kruskal-Wallis test was used to detect statistical significance ($p < 0.05$).

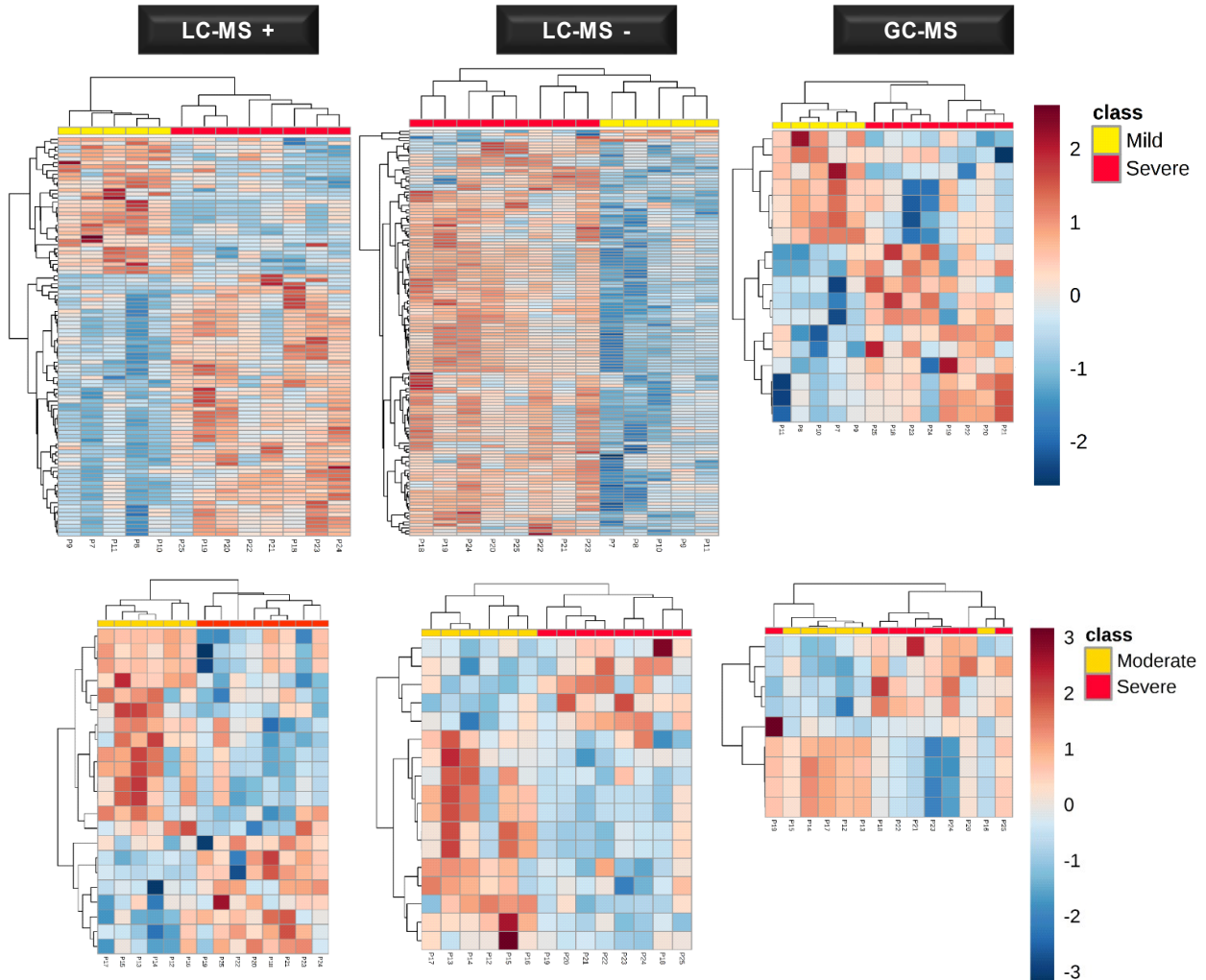
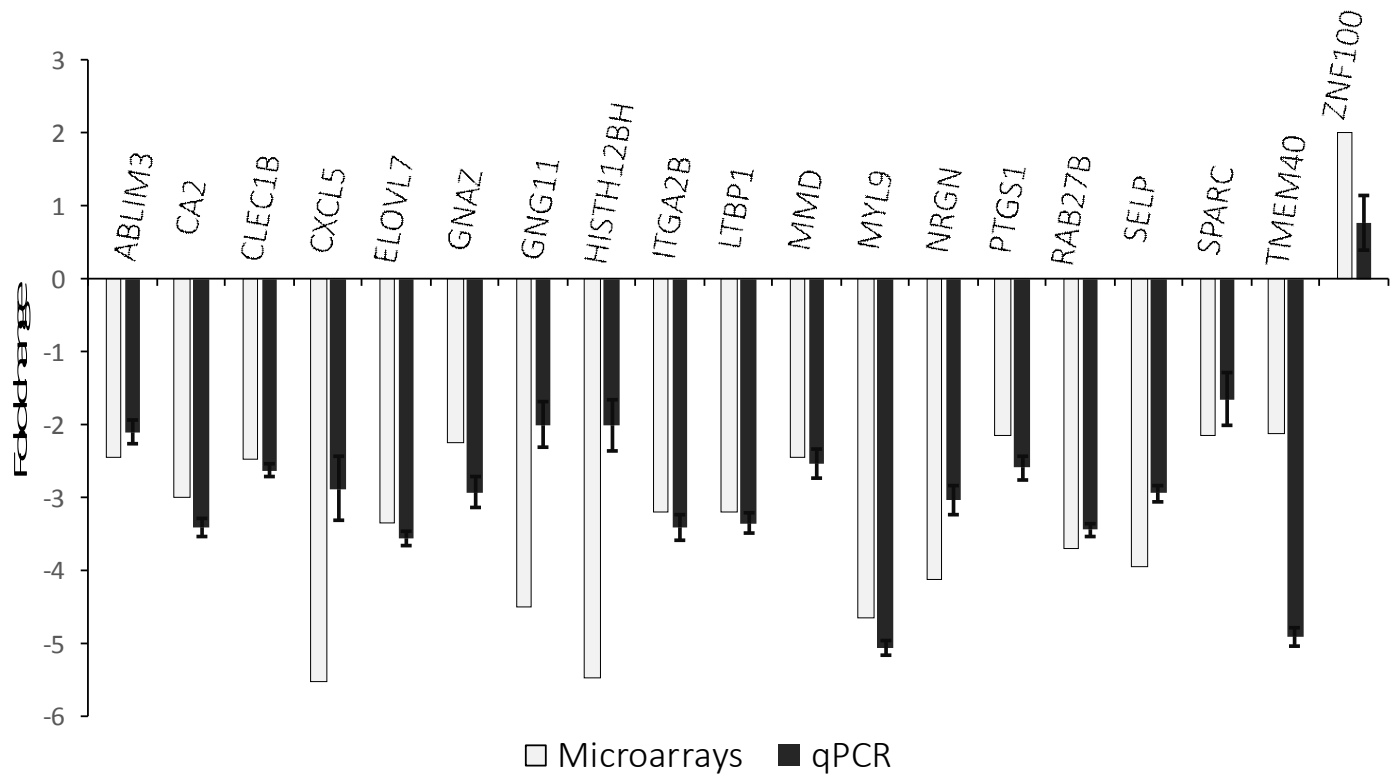


Figure 4S. Validation of microarray data by qPCR using 2^{-ΔΔCT} method. Microarray results (white) compared to qPCR results (black). Standard deviation bars are represented in qPCR results.



P.25	Severe profilin-allergic	2.24	0	2.42	0	0	0	0	0	0	0	1.62	0	0	1.30
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Table 2S. Patient information.

Patient	Age/Sex	Self-reported Food Allergy	Self-Reported Reactions	Reaction to Profilin Challenge	Rescue Medication ^{&}	Clinical Status*
P.1	36/F	None	None	None	None	Non-allergic
P.2	34/M	None	None	None	None	Non-allergic
P.3	29/F	None	None	None	None	Non-allergic
P.4	35/F	None	None	None	None	Non-allergic
P.5	27/F	None	None	None	None	Non-allergic
P.6	38/F	None	None	None	None	Non-allergic
P.7	47/F	B, T	OAS,EM	None	None	Non-severe profilin-allergic
P.8	19/F	M, Pe, S	OAS	OAS	None	Non-severe profilin-allergic
P.9	41/F	P, Pe, Ap,Che, K, B, Av, Pi, Ma, Pn, M, W	OAS,RESP	RESP	Salbutamol	Non-severe profilin-allergic
P.10	53/M	None	None	OP (2/10)	None	Non-severe profilin-allergic
P.11	29/M	None	None	OP	Cetirizine	Non-severe profilin-allergic
P.12	34/F	S, A	ERY	OAS	None	Non-severe profilin-allergic
P.13	37/M	None	None	none	None	Non-severe profilin-allergic
P.14	44/F	P, Pe, S, Q, K, B, Av, Pi, Cn, O, F, Wa, Au, T, Cu, M	OAS	OP (8/10)	None	Non-severe profilin-allergic
P.15	44/F	L	BS, EP, HY, NA, RHI, UR, EM	DSP, OAS	IV Anti-histamine and Corticoid	Non-severe profilin-allergic
P.16	28/F	M, W, Che	OAS, DY	CS, OAS	None	Non-severe profilin-allergic
P.17	38/M	K, Le, Ma, M, Pi, S	OAS	OP, CG, DSP	Cetirizine	Non-severe profilin-allergic
P.18	25/F	M, W	ABP, OAS, DY, DSP, EP, EU, PB, NA, EM	OAS, DSP, LA, NA	IV Anti - histaminic, Corticoid, IM Adrenalin	Severe profilin-allergic
P.19	44/F	M	OAS, ERY, CG, PB, EP	LA, OAS, DSP, NA	IM Adrenalin, IV Anti - histamine	Severe profilin-allergic
P.20*	26/F	L, M, T	BS, EU	Not carried out		Severe profilin-

						allergic
P.21	31/F	M, W	OAS	LA	None	Non-severe profilin-allergic
P.22	26/M	M, W, B	OAS, LA	None	None	Non-severe profilin-allergic
P.23	20/F	Ma, T	OAS	EU, DSP	IM Adrenalin, IV Actocortine, Dexchlorpheniramine maleate	Severe profilin-allergic
P.24	22/M	M, S	BS, OAS	BS, EU, DSP	IM Adrenalin, IV Actocortine, Dexchlorpheniramine maleate	Severe profilin-allergic
P.25*	41/F	M	BS, EU, UR	Not carried out		Severe profilin-allergic

Table 3S. Genes selected for validation studies by qPCR.

GENE SYMBOL	DESCRIPTION
CXCL5	Chemokine (C-X-C motif) ligand 5.
HIST1H2BH	Histone cluster 1, H2bh.
MYL9	Myosin, light chain 9, regulatory.
TREML1	Triggering receptor expressed on myeloid cells-like 1.
GNG11	Guanine nucleotide binding protein (G protein), gamma 11.
NRGN	Neurogranin (protein kinase C substrate, RC3).
SELP	Selectin P (granule membrane protein 140kDa, antigen CD62).
RAB27B	Member RAS oncogene family.
ELOVL7	ELOVL fatty acid elongase 7; ELOVL family member 7 .
CLU	Clusterin.
LTBP1	Latent transforming growth factor beta binding protein 1.
ITGA2B	Integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41).
CA2	Carbonic anhydrase II.
CLEC1B	C-type lectin domain family 1, member B.
ABLIM3	Actin binding LIM protein family, member 3.
MMD	Monocyte to macrophage differentiation-associated.
GNAZ	Guanine nucleotide binding protein (G protein), alpha z polypeptide.
SPARC	Cecreted protein, acidic, cysteine-rich (osteonectin).
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase).
TMEM40	Transmembrane protein 40
ZNF100	Zinc finger protein 100

NOTE: Description source was Transcriptome Analysis Console (annotation library file: HuGene-2_1-st-v1.na36.hg19.transcript.csv)

Table 4S. Results from the OPLS-DA prediction model.

Patient Code	Clinical Status	Assigned group*	Predicted score*	Assigned Group*	Predicted Group (Severe)	Classification Group
P.1	Non-allergic	1	1.006	0	-0.006	Non-allergic
P.2	Non-allergic	1	1.026	0	-0.026	Non-allergic
P.3	Non-allergic	1	1.024	0	-0.024	Non-allergic
P.4	Non-allergic	1	0.904	0	0.096	Non-allergic
P.5	Non-allergic	1	1.024	0	-0.024	Non-allergic
P.6	Non-allergic	1	0.994	0	0.006	Non-allergic
P.7	Non-severe profilin-allergic	–	0.756	–	0.244	Mild
p.8	Non-severe profilin-allergic	–	0.740	–	0.260	Mild
p.9	Non-severe profilin-allergic	–	0.912	–	0.088	Mild
P.10	Non-severe profilin-allergic	–	0.864	–	0.136	Mild
P.11	Non-severe profilin-allergic	–	0.812	–	0.188	Mild
P.12	Non-severe profilin-allergic	–	0.260	–	0.740	Moderate
P.13	Non-severe profilin-allergic	–	0.531	–	0.469	Moderate
P.14	Non-severe profilin-allergic	–	0.580	–	0.420	Moderate
P.15	Non-severe profilin-allergic	–	0.516	–	0.484	Moderate
P.16	Non-severe profilin-allergic	–	0.358	–	0.642	Moderate
P.17	Non-severe profilin-allergic	–	0.651	–	0.349	Moderate
P.21	Non-severe profilin-allergic	–	0.080	–	0.920	Severe
P.22	Non-severe profilin-allergic	–	0.116	–	0.884	Severe
P.18	Severe profilin-allergic	0	-0.042	1	1.042	Severe
P.19	Severe profilin-allergic	0	-0.054	1	1.054	Severe
P.20	Severe profilin-allergic	0	0.035	1	0.965	Severe
P.23	Severe profilin-allergic	0	0.043	1	0.957	Severe
P.24	Severe profilin-allergic	0	-0.008	1	1.008	Severe
P.25	Severe profilin-allergic	0	0.048	1	0.952	Severe

*Non-allergic subjects; &Severe profilin-allergic patients; values used to build the OPLS-DA model.

Table 5S. Significant metabolites using Kruskal-Wallis test with a Benjamini-Hochberg (BH) correction found in the three platforms; LC-MS+, LC-MS– and GC-MS.

No	Technique	Compound	<i>m/z</i> (Da) (LC-MS) / ion target (GC-MS)	Mass (Da)	RT (min)	Formula	Error (ppm)	Adduct	C V	<i>p</i> value	<i>p</i> BH
1	LC-MS+	Acetylcarnitine*	204.1221	203.1152	0.69	C9 H17 N O4	-4.4	M+H	3	0.039	0.046
2	GC-MS	Adenosine	–	236.0	23.85	C10 H13 N5 O4	–	–	16	0.021	0.040
3	GC-MS	Aminoisobutyric acid	–	218.0	12.48	C4 H9 N O2	–	–	22	0.041	0.045
4	GC-MS	Aminomalonic acid	–	320.1	12.47	C3 H5 N O4	–	–	24	0.045	0.045
5	LC-MS+	C17 Sphinganine ^{&}	288.2891	287.2802	11.24	C17 H37 N O2	-1.7	M+H	2	0.035	0.043
6	LC-MS+	C17 Sphinganine*	288.2894	287.2820	11.98	C17 H37 N O2	-0.7	M+H	2	0.081	0.104
7	LC-MS+	Carnitine*	162.1119	161.1046	0.69	C7 H15 N O3	-3.1	M+H	2	0.017	0.042
8	LC-MS+	Chenodeoxycholic acid glycine conjugate	450.3215	449.3139	12.45	C26 H	0.4	M+H	1	0.232	0.232

						43 N O5						
9	GC-MS	Cysteine	–	220.0	13.58	C3 H7 N O2 S	–	–	1 1	0.016	0.040	
10	GC-MS	D-Allose	–	319.2	17.54	C6 H1 2O 6	–	–	7	0.050	0.106	
11	GC-MS	D-Glucose/D- Altrose/D- Galactose /Talose	–	319.2	17.34	C6 H1 2O 6	–	–	7	0.037	0.045	
12	LC-MS-	Dihydroxycholanoic acid	391.2856	392.2924	10.89	C2 4H 4O O4	0.3	M-H	5	0.099	0.106	
13	GC-MS	D-Lyxosylamine	–	103.0	14.78	C5 H1 1N O4	–	–	8	0.036	0.045	
14	GC-MS	D-Mannose /D-Allose	–	319.2	17.22	C6 H1 2O	–	–	1 0	0.011	0.040	
15	GC-MS	D-Mannitol/D- Mannose/D-Sorbitol	–	319.0	17.75	C6 H1 4O 6	–	–	1 7	0.058	0.106	
16	GC-MS	Glyceric acid	–	189.1	10.62	C3 H6 O4	–	–	5	0.113	0.147	
17	LC-MS+	Hexanoylcarnitine*	260.1861	259.1790	0.99	C1 3H 25 N O4	1.9	M+H	1	0.196	0.204	
18	GC-MS	Hypoxanthine	–	265.1	16.41	C5 H2 N4	–	–	1 5	0.015	0.040	

						O						
19	GC-MS	L-Sorbose/Fructose	–	103.0	17.16	C6 H1 2O 6	–	–	7	0.009	0.040	
20	GC-MS	Lactamide	–	100.0	7.94	C3 H7 N O2	–	–	2 1	0.287	0.295	
21	GC-MS	Lactic acid	–	117.1	6.81	C3 H6 O3	–	–	4	0.021	0.040	
22	GC-MS	Lauric acid	–	117.0	14.71	C1 2H 24 O2	–	–	1 5	0.085	0.135	
23	GC-MS	Leucine	–	158.1	9.83	C6 H1 3N O2	–	–	1 2	0.097	0.139	
24	LC-MS+	Leucyl-proline*	229.1544	228.1471	0.70	C1 1H 20 N2 O3	-0.9	M+H	3	0.051	0.081	
25	GC-MS	L-Methionine	–	61.0	11.75	C5 H1 1N O2 S	–	–	1 1	0.057	0.106	
26	LC-MS-	LysoPC (P-16:0)/LysoPC (O-16:1)	524.3348	479.3366	21.44	C2 4H 50 N O6 P	-2.1	M+COOH	4	0.011	0.027	
27	LC-MS-	LysoPC (P-18:0)/LysoPC (O-18:1)	552.3658	553.3793	26.01	C2 6H 54 N O6	-2.6	M+COOH	1 0	0.018	0.028	

						P						
28	LC-MS-	LysoPC (P-18:0)/LysoPC (O-18:1)	552.366	553.3747	22.23	C2 6H 54 N O6 P	-2.2	M+COOH	3	0.021	0.028	
29	LC-MS-	LysoPC (P-18:1)/LysoPC (O-18:2)	550.3513	551.3602	22.30	C2 6H 52 N O6 P	-0.2	M+COOH	4	0.048	0.049	
30	LC-MS-	LysoPC(14:0)*	556.3247	557.3327	16.11	C2 2H 46 N O7 P	-2.1	M+90.0320	1 1	0.021	0.028	
31	LC-MS-	LysoPC(16:0)*	540.3311	541.3386	19.44	C2 4H 50 N O7 P	0.8	M+COOH	3	0.005	0.027	
32	LC-MS-	LysoPC(16:0)*	530.3023	531.3102	20.21	C2 4H 50 N O7 P	0.8	M+Cl	4	0.016	0.027	
33	LC-MS-	LysoPC(18:0)*	558.3314	559.3407	23.76	C2 6H 54 N O7 P	-3.4	M+Cl	8	0.029	0.033	
34	LC-MS-	LysoPC(18:0)*	636.3488	637.3572	24.59	C2 6H 52 N	1.3	M+112.955 5	5	0.013	0.027	

						O7 P					
35	LC-MS-	LysoPC(18:1)*	610.3716	611.3799	21.12	C2 6H 52 N O7 P	-1.9	M+90.0320	8	0.009	0.027
36	LC-MS-	LysoPC(18:1)*	610.3708	611.3700	20.43	C2 6H 52 N O7 P	-3.5	M+90.0320	9	0.023	0.029
37	LC-MS-	LysoPC(18:2)	554.3006	555.3098	17.88	C2 6H 50 N O7 P	-2.5	M+Cl	9	0.011	0.027
38	LC-MS+	LysoPC(19:0)*	538.3879	537.3828	26.98	C2 7H 56 N O7 P	2.4	M+H	9	0.040	0.046
39	LC-MS+	LysoPC(20:0)*	552.4027	551.3953	29.50	C2 8H 58 N O7 P	0.7	M+H	6	0.047	0.049
40	LC-MS-	LysoPC(20:1)	594.377	595.3855	25.12	C2 8H 56 N O7 P	-1.1	M+COOH	4	0.019	0.028
41	LC-MS+	LysoPC(20:1)	550.3868	549.3790	25.13	C2 8H 56 N	0.4	M+H	2	0.090	0.110

						O7 P						
42	LC-MS-	LysoPC(20:2)	592.3626	593.3622	21.49	C2 8H 54 N O7 P	1.1	M+COOH	1 0	0.042	0.044	
43	LC-MS+	LysoPC(20:2)	548.371	547.3632	22.18	C2 8H 54 N O7 P	0.0	M+H	1 0	0.004	0.042	
44	LC-MS-	LysoPC(20:4)	656.3191	657.3357	17.99	C2 8H 50 N O7 P	1.8	M+112.955 5	1 9	0.019	0.028	
45	LC-MS-	LysoPC(22:6)	612.3382	613.2694	19.43	C3 0H 50 N O7 P	13.2	M+COOH	4	0.004	0.027	
46	LC-MS-	LysoPC(24:6)	640.3678	641.3887	21.12	C3 2H 54 N O7 P	9.7	M+COOH	7	0.006	0.027	
47	LC-MS+	LysoPC(O-16:0)	482.3601	481.3527	21.43	C2 4H 52 N O6 P	-0.6	M+H	4	0.015	0.042	
48	LC-MS-	LysoPC(O-18:0)	554.3844	555.3916	26.20	C2 6H 56 N	3.3	M+COOH	9	0.007	0.027	

						O6 P					
49	LC-MS+	LysoPE(18:0)*	464.3137	463.3080	21.83	C2 3H 48 N O7 P	0.4	M+H-H2O	8	0.005	0.042
50	LC-MS+	LysoPE(18:0)*	482.3247	481.3172	24.03	C2 3H 48 N O7 P	1.5	M+H	2	0.019	0.042
51	LC-MS-	LysoPE(20:2)	504.3088	505.3172	18.53	C2 5H 48 N O7 P	-1.6	M-H	7	0.013	0.027
52	LC-MS-	LysoPE(20:2)	504.3088	505.3176	17.88	C2 5H 48 N O7 P	-1.6	M-H	1 6	0.018	0.028
53	LC-MS+	LysoPE(P-16:0)	438.2983	437.2904	20.96	C2 1H 44 N O6 P	1.1	M+H	5	0.008	0.042
54	LC-MS+	LysoPE(P-18:0)/LysoPE (O-18:1)	466.3299	465.3239	25.28	C2 3H 48 N O6 P	1.7	M+H	7	0.002	0.042
55	LC-MS-	LysoPS(18:0)*	524.2981	525.3077	27.15	C2 4H 48 N	-2.7	M-H	7	0.019	0.028

						O9 P					
56	GC-MS	Methyl-β-D-Galactopyranoside	–	217.0	16.88	C6 H1 2O 6	–	–	3	0.133	0.150
57	GC-MS	Myo-Inositol	–	217.0	19.26	C6 H1 2O 6	–	–	1 7	0.264	0.280
58	GC-MS	Myristic acid	–	285.0	16.88	C1 4H 28 O2	–	–	5	0.056	0.056
59	GC-MS	Oleic acid	–	117.0	20.44	C1 8H 34 O2	–	–	7	0.207	0.207
60	GC-MS	Oxalic acid	–	147.1	7.96	C2 H2 O4	–	–	2 7	0.326	0.326
61	GC-MS	Palmitic acid	–	117.0	18.85	C1 6H 32 O2	–	–	7	0.111	0.147
62	GC-MS	Phenylalanine	–	218.1	14.41	C9 H1 1N O2	–	–	1 8	0.024	0.040
63	GC-MS	Pyruvic acid	–	174.1	6.64	C3 H4 O3	–	–	3	0.016	0.040
64	GC-MS	Serine	–	116.1	9.65	C3 H7 N O3	–	–	1 6	0.124	0.148
65	LC-MS-	Sphinganine-1-phosphate*	380.257	381.2643	16.59	C1 8H 40 N O5 P	-0.5	M-H	6	0.100	0.106

66	LC-MS+	Sphingosine*	300.2893	299.2801	15.22	C1 8H 37 N O2	-1.0	M+H	6	0.034	0.043
67	LC-MS-	Sphingosine-phosphate oxidized (Non described)	376.2253	377.2336	13.15	C1 8H 36 N O5 P	-1.6	M-H	1	0.163	0.163
68	LC-MS-	Sphingosine-1-phosphate*	378.2409	379.2489	15.69	C1 8H 38 N O5 P	-1.8	M-H	2	0.112	0.116
69	GC-MS	Tagatose	–	307.2	17.16	C6 H1 2O 6	–	–	9	0.063	0.106
70	GC-MS	Trihydroxybutyric acid	–	292.0	13.58	C4 H8 O5	–	–	8	0.005	0.040
71	LC-MS+	Unknown-Sphingolipid related	304.2997	303.2922	15.61	C2 1H 38 N	-0.3	M+H	1 3	0.012	0.042
72	GC-MS	Urea	–	189.1	9.43	CH 4N 2O	–	–	8	0.146	0.160
73	GC-MS	Valine	–	144.1	9.04	C5 H1 1N O2	–	–	6	0.043	0.045
74	LC-MS+	Xestoaminol C	230.2475	229.2400	10.20	C1 4H 31 N O	-1.3	M+H	8	0.058	0.087

NOTE: &Same mass, m/z and fragmentation pattern as the standard.

*ID confirmed with commercial standard.

Those p -values and p_{BH} -values that are not highlighted in green were significant in the pairwise comparison using Kruskal-Wallis test with a Benjamini-Hochberg (BH) correction. RT= Retention time.

Table 6S. Pairwise comparisons showing the significance and % of change among experimental groups using U-Mann Whitney test.

No	Technique	Compound	<i>m/z</i> (Da) (LC-MS) / ion target (GC-MS)	Mass (Da)	RT (min)	Non allergic vs Moderate		Mild vs Moderate		Mild vs Severe		Moderate vs Severe	
						<i>p</i> value	<i>p</i> value	% Severe	% Moderate	% Severe	<i>p</i> value	% Severe	<i>p</i> value
1	LC-MS+	Acetylcarnitine*	204.1221	203.1152	0.69	0.429	0.009	-33.9	-27.2	-18.5	0.127	12.0	0.852
2	GC-MS	Adenosine	–	236.0	23.85	1.000	0.028	781.0	294.3	322.0	0.048	7.0	0.982
3	GC-MS	Aminoisobutyric acid	–	218.0	12.48	0.082	0.240	14.1	114.4	97.9	0.065	-7.7	0.414
4	GC-MS	Aminomalonic acid	–	320.1	12.47	0.126	0.394	11.3	105.8	91.4	0.045	-7.0	0.345
5	LC-MS+	C17 Sphinganine*	288.2891	287.2802	11.24	0.247	1.000	-81.4	-44.7	-81.8	0.006	-67.1	0.059
6	LC-MS+	C17 Sphinganine*	288.2894	287.2820	11.98	0.429	0.485	-73.8	-32.5	-74.2	0.019	-61.8	0.043
7	LC-MS+	Carnitine*	162.1119	161.1046	0.69	0.792	0.041	-34.7	-17.6	-27.5	0.019	-12.0	0.282
8	LC-MS+	Chenodeoxycholic acid glycine conjugate	450.3215	449.3139	12.45	0.662	0.589	-63.1	-16.4	-44.8	0.045	-34.0	0.414
9	GC-MS	Cysteine	–	220.0	13.58	0.004	0.589	-42.5	78.6	23.0	0.284	-31.2	0.142
10	GC-MS	D-Allose	–	319.2	17.54	0.662	0.699	-33.0	-1.3	-32.7	0.065	-31.8	0.029
11	GC-MS	D-Glucose/D-Altrose/D- Galactose /Talose	–	319.2	17.34	0.931	0.818	-31.3	-5.1	-32.1	0.030	-28.5	0.029
12	LC-MS-	Dihydroxycholanoic acid	391.2856	392.2924	10.89	0.126	0.041	-71.2	-27.0	21.3	0.284	66.2	0.345
13	GC-MS	D-Lyxosylamine	–	103.0	14.78	1.000	0.026	6.0	-34.7	-4.4	0.284	46.3	0.043
14	GC-MS	D-Mannose/D-Allose	–	319.2	17.22	0.247	0.310	-34.4	-24.3	-43.0	0.006	-24.7	0.142
15	GC-MS	D-Mannitol/D- Mannose/D-Sorbitol	–	319.0	17.75	0.030	0.041	242.1	-63.6	-55.5	0.943	22.3	0.950
16	GC-MS	Glyceric acid	–	189.1	10.62	0.126	0.394	-12.8	26.4	38.3	0.019	9.5	0.345
17	LC-MS+	Hexanoylcarnitine*	260.1861	259.1790	0.99	0.931	0.041	-19.2	-33.6	-20.0	0.524	20.6	0.662
18	GC-MS	Hypoxanthine	–	265.1	16.41	0.662	0.132	388.8	88.2	321.9	0.019	124.1	0.142
19	GC-MS	L- Sorbose/Fructose	–	103.0	17.16	0.247	0.240	-32.5	-21.5	-40.8	0.011	-24.5	0.142
20	GC-MS	Lactamide	–	100.0	7.94	0.329	0.589	4.4	39.6	18.6	0.001	-15.1	0.950
21	GC-MS	Lactic acid	–	117.1	6.81	0.126	0.394	56.8	-8.4	25.3	0.093	36.8	0.043

22	GC-MS	Lauric acid	–	117.0	14.71	0.247	0.041	-40.5	-42.7	-15.3	0.724	47.7	0.081
23	GC-MS	Leucine	–	158.1	9.83	0.030	0.394	47.2	-36.7	-29.7	0.222	11.1	0.662
24	LC-MS+	Leucyl-proline*	229.1544	228.1471	0.70	0.030	0.937	-8.4	-22.9	-31.1	0.030	-10.7	0.282
25	GC-MS	L-Methionine	–	61.0	11.75	0.792	0.485	41.3	41.3	56.9	0.019	11.0	0.228
26	LC-MS-	LysoPC (P-16:0)/LysoPC (O-16:1)	524.3348	479.3366	21.44	0.429	0.180	32.1	56.9	60.6	0.002	2.4	0.950
27	LC-MS-	LysoPC (P-18:0)/LysoPC (O-18:1)	552.3658	553.3793	26.01	0.126	0.394	16.3	121.2	96.8	0.002	-11.0	0.755
28	LC-MS-	LysoPC (P-18:0)/LysoPC (O-18:1)	552.366	553.3747	22.23	0.329	0.485	29.2	47.9	69.3	0.003	14.5	0.228
29	LC-MS-	LysoPC (P-18:1)/LysoPC (O-18:2)	550.3513	551.3602	22.30	0.429	0.485	19.6	55.1	54.7	0.011	-0.2	0.950
30	LC-MS-	LysoPC(14:0)*	556.3247	557.3327	16.11	0.662	0.004	44.3	12.9	25.7	0.284	11.2	0.573
31	LC-MS-	LysoPC(16:0)*	540.3311	541.3386	19.44	0.082	0.132	17.6	45.7	44.8	0.002	-0.7	0.662
32	LC-MS-	LysoPC(16:0)*	530.3023	531.3102	20.21	0.030	0.310	5.0	17.3	17.1	0.006	-0.2	0.755
33	LC-MS-	LysoPC(18:0)*	558.3314	559.3407	23.76	0.082	0.699	12.9	77.1	75.4	0.011	-0.9	0.662
34	LC-MS-	LysoPC(18:0)*	636.3488	637.3572	24.59	0.052	0.180	8.4	50.0	45.8	0.006	-2.8	0.950
35	LC-MS-	LysoPC(18:1)*	610.3716	611.3799	21.12	0.247	0.180	25.8	45.9	51.0	0.002	3.5	0.755
36	LC-MS-	LysoPC(18:1)*	610.3708	611.3700	20.43	0.429	0.310	24.4	34.3	43.9	0.003	7.2	0.491
37	LC-MS-	LysoPC(18:2)	554.3006	555.3098	17.88	0.017	0.589	14.8	56.1	68.0	0.003	7.7	0.573
38	LC-MS+	LysoPC(19:0)*	538.3879	537.3828	26.98	0.126	1.000	6.1	56.4	69.0	0.011	8.1	0.491
39	LC-MS+	LysoPC(20:0)*	552.4027	551.3953	29.50	0.329	0.240	13.9	66.2	47.0	0.030	-11.5	0.491
40	LC-MS-	LysoPC(20:1)	594.377	595.3855	25.12	0.247	0.180	25.9	73.4	66.2	0.011	-4.2	0.950
41	LC-MS+	LysoPC(20:1)	550.3868	549.3790	25.13	0.662	0.310	22.5	39.7	35.8	0.011	-2.8	1.000
42	LC-MS-	LysoPC(20:2)	592.3626	593.3622	21.49	0.537	0.394	41.3	42.9	70.8	0.011	19.5	0.181
43	LC-MS+	LysoPC(20:2)	548.371	547.3632	22.18	0.537	0.180	42.6	29.8	57.6	0.019	21.4	0.020
44	LC-MS-	LysoPC(20:4)	656.3191	657.3357	17.99	0.931	0.132	36.4	42.7	50.1	0.006	5.2	0.491
45	LC-MS-	LysoPC(22:6)	612.3382	613.2694	19.43	0.082	0.041	15.7	32.7	32.5	0.003	-0.1	0.852
46	LC-MS-	LysoPC(24:6)	640.3678	641.3887	21.12	0.004	0.394	15.3	52.9	57.6	0.002	3.1	0.755
47	LC-MS+	LysoPC(O-16:0)	482.3601	481.3527	21.43	0.429	0.310	32.9	35.8	53.3	0.006	12.9	0.181
48	LC-MS-	LysoPC(O-18:0)	554.3844	555.3916	26.20	0.126	0.180	37.4	78.7	91.2	0.002	7.0	0.755
49	LC-MS+	LysoPE(18:0)*	464.3137	463.3080	21.83	0.931	0.004	56.2	53.5	42.5	0.030	-7.2	0.755
50	LC-MS+	LysoPE(18:0)*	482.3247	481.3172	24.03	0.662	0.009	23.6	28.7	24.8	0.127	-3.1	0.573

51	LC-MS-	LysoPE(20:2)	504.3088	505.3172	18.53	0.017	1.000	5.4	62.7	63.6	0.002	0.5	0.491
52	LC-MS-	LysoPE(20:2)	504.3088	505.3176	17.88	0.052	0.699	4.9	74.7	70.7	0.002	-2.3	1.000
53	LC-MS+	LysoPE(P-16:0)	438.2983	437.2904	20.96	0.792	0.002	49.9	35.9	32.8	0.093	-2.2	0.852
54	LC-MS+	LysoPE(P-18:0)/LysoPE (O-18:1)	466.3299	465.3239	25.28	0.792	0.004	46.9	57.8	45.1	0.006	-8.0	0.414
55	LC-MS-	LysoPS(18:0)*	524.2981	525.3077	27.15	0.662	0.041	86.5	66.7	75.8	0.045	5.5	0.573
56	GC-MS	Methyl- β -D-Galactopyranoside	–	217.0	16.88	1.000	0.180	11.8	-31.2	11.1	1.000	61.5	0.029
57	GC-MS	Myo-Inositol	–	217.0	19.26	0.030	0.394	-0.8	0.7	-18.5	0.222	-19.1	0.414
58	GC-MS	Myristic acid	–	285.0	16.88	0.082	0.041	-27.3	-16.2	1.6	0.943	21.3	0.181
59	GC-MS	Oleic acid	–	117.0	20.44	0.247	0.589	-2.1	-1.6	63.0	0.127	65.7	0.043
60	GC-MS	Oxalic acid	–	147.1	7.96	0.429	0.485	-0.9	49.1	19.9	0.001	-19.6	0.852
61	GC-MS	Palmitic acid	–	117.0	18.85	0.177	0.699	2.0	10.4	48.2	0.045	34.3	0.043
62	GC-MS	Phenylalanine	–	218.1	14.41	0.017	0.818	20.7	-37.3	-37.7	0.003	-0.6	0.950
63	GC-MS	Pyruvic acid	–	174.1	6.64	0.052	0.818	-31.7	-55.9	-66.4	0.003	-23.9	0.228
64	GC-MS	Serine	–	116.1	9.65	0.052	0.818	-5.6	60.1	61.3	0.093	0.7	1.000
65	LC-MS-	Sphinganine-1-phosphate*	380.257	381.2643	16.59	0.177	0.132	41.2	-1.2	5.4	0.354	6.7	0.491
66	LC-MS+	Sphingosine*	300.2893	299.2801	15.22	0.052	0.026	78.3	1.5	-12.4	0.622	-13.6	0.950
67	LC-MS-	Sphingosine-phosphate oxidized related (Non described)	376.2253	377.2336	13.15	0.537	0.589	20.5	-0.6	13.1	0.171	13.8	0.282
68	LC-MS-	Sphingosine-1-phosphate*	378.2409	379.2489	15.69	0.662	0.310	33.0	4.2	19.0	0.284	14.2	0.228
69	GC-MS	Tagatose	–	307.2	17.16	0.792	0.180	-33.3	-58.4	-64.6	0.065	-15.0	0.414
70	GC-MS	Trihydroxybutyric acid	–	292.0	13.58	0.004	0.485	-43.7	100.9	45.0	0.045	-27.8	0.108
71	LC-MS+	Unknown-Sphingolipid	304.2997	303.2922	15.61	0.429	0.180	-47.6	-47.5	-61.1	0.006	-25.9	0.228
72	GC-MS	Urea	–	189.1	9.43	0.931	0.394	37.0	16.3	33.2	0.040	14.5	0.266
73	GC-MS	Valine	–	144.1	9.04	0.030	0.394	36.0	-34.9	-35.7	0.030	-1.3	0.950
74	LC-MS+	Xestoaminol C	230.2475	229.2400	10.20	0.052	0.485	-71.9	210.6	-29.0	0.435	-77.1	0.573
Number of significant metabolites						12	17			49	9		

NOTE: *ID confirmed with commercial standard. Cells with a U-Mann Whitney p -value less than 0.05 are highlighted in red. The percentage of change was colored in red for positive increment and in blue for decrease. Significant metabolites from “Non allergic vs Mild” $n=12$; Significant metabolites from “Non allergic vs Moderate” $n=17$; Significant metabolites from “Non allergic vs Severe” $n=32$; Significant metabolites from “Mild vs Moderate” $n=29$; Significant metabolites from “Mild vs Severe” $n=49$; Significant metabolites from “Moderate vs Severe” $n=9$.

Table 7S. Top 100 genes used to perform heatmap among all experimental groups. Genes are ranked by p-value.

N	Gene Symbol*	Fold Change	ANOVA p-value	Comparison
1	RNA5SP395	-3.19	0.000002	Mild Vs Severe
2	DDX11L2; DDX11L9; DDX11L10; DDX11L1; DDX11L5; DDX11L12; DDX11L16; DDX11L11	4.97	0.000003	Control Vs Severe
3	SELP	3.94	0.000008	Control Vs Severe
4	GUSBP3	-4.72	0.000016	Mild Vs Severe
5	NRGN	4.23	0.000024	Control Vs Severe
6	NPIPB3	-3.14	0.000041	Mild Vs Severe
7	TREML1	4.58	0.000042	Control Vs Severe
8	HIST1H2BH	5.99	0.000044	Moderate Vs Severe
9	MYL9	4.58	0.000054	Control Vs Severe
10	SDPR	4.43	0.000074	Control Vs Severe
11	SPDYA	-4.51	0.000075	Mild Vs Severe
12	RNU5E-4P	10.68	0.000091	Moderate Vs Severe
13	HIST1H2BH	6.65	0.000098	Control Vs Severe
14	SCARNA5	-9.2	0.000138	Mild Vs Severe
15	TUBB1	3.68	0.000163	Control Vs Severe
16	RAB27B	3.58	0.000241	Control Vs Severe
17	DDX11L10	3.82	0.00026	Control Vs Severe
18	C15orf54	3.72	0.00029	Control Vs Severe
19	GPR97	3.5	0.000301	Moderate Vs Severe
20	CXCR2P1	3.6	0.000313	Control Vs Severe
21	RP11-33B1.3	-3.53	0.000644	Mild Vs Severe
22	SCARNA5	-7.55	0.000672	Moderate Vs Severe
23	RNA5SP85	-4.8	0.000699	Mild Vs Severe
24	SH3BGRL2	3.46	0.000719	Control Vs Severe
25	RNA5SP199	-13.45	0.000737	Mild Vs Severe
26	NRGN	3.51	0.000775	Moderate Vs Severe
27	MT-TG	4.53	0.000809	Moderate Vs Severe
28	SNORA38B	-9.78	0.00096	Mild Vs Severe
29	MIR644A	-4.16	0.001124	Mild Vs Severe
30	MYL9	3.92	0.001144	Moderate Vs Severe
31	RNU7-181P	-3.17	0.001169	Mild Vs Severe
32	MIR3945	7.9	0.001208	Moderate Vs Severe
33	TREML1	3.95	0.001228	Moderate Vs Severe
34	SNORA14A	-4.69	0.001242	Mild Vs Severe
35	CXCL5	4.9	0.001296	Control Vs Severe
36	RNVU1-19	6.65	0.001315	Moderate Vs Severe

37	SNORA9; SNHG15; LOC101928927	-3.76	0.001347	Mild Vs Severe
38	ITGB3; RP11-290H9.2	4.86	0.001475	Control Vs Severe
39	MT-TR	4.36	0.001694	Control Vs Severe
40	MT-TG	5.86	0.001763	Control Vs Severe
41	LOC100272216; GUSBP1	-3.73	0.001884	Mild Vs Severe
42	SNORA16B	-3.57	0.001946	Mild Vs Severe
43	RNA5SP199	-9.54	0.002237	Moderate Vs Severe
44	RNU6-341P	-3.3	0.00233	Mild Vs Severe
45	SNORA20	-3.52	0.00237	Mild Vs Severe
46	GP1BA	3.46	0.002509	Moderate Vs Severe
47	RNA5SP19	-4.39	0.002641	Mild Vs Severe
48	PPBP	3.86	0.002918	Moderate Vs Severe
49	TRAJ14	3.92	0.002966	Control Vs Mild
50	HIST1H3H; HIST1H3A; HIST1H3D; HIST1H3C; HIST1H3E; HIST1H3I; HIST1H3G; HIST1H3J; HIST1H3B; HIST1H3F	4.37	0.003249	Control Vs Severe
51	CXCL8; IL8	3.54	0.003439	Moderate Vs Severe
52	APOLD1	-3.86	0.003466	Mild Vs Severe
53	RNU5E-4P	-4.86	0.003774	Mild Vs Moderate
54	SNORA84; MIR3651	-4.1	0.00391	Mild Vs Severe
55	RASA4B; RASA4	-3.8	0.004318	Mild Vs Moderate
56	RNU1-59P	3.56	0.004614	Moderate Vs Severe
57	RNU5D-1	-4.74	0.004885	Control Vs Moderate
58	SNORA14A	-3.28	0.005002	Control Vs Severe
59	MYL9	3.69	0.00533	Mild Vs Severe
60	RNVU1-3	-3.24	0.00631	Mild Vs Moderate
61	AP001189.4	3.84	0.006352	Control Vs Severe
62	MIR548C; MIR548Z	-3.06	0.006363	Mild Vs Severe
63	RNU4ATAC11P	-3.42	0.006621	Mild Vs Severe
64	HIST1H2BH	4.07	0.00722	Mild Vs Severe
65	RNU6-135P	-3.19	0.007274	Mild Vs Severe
66	RNU5F-1	6.68	0.007498	Moderate Vs Severe
67	TRAJ19	5.9	0.008348	Control Vs Mild
68	RNU5F-1	-3.83	0.008493	Control Vs Moderate
69	TREML1	3.87	0.009201	Mild Vs Severe
70	SNORA14B	-3.5	0.009239	Mild Vs Severe
71	C15orf54	3.59	0.00967	Control Vs Mild
72	MIR3945	-8.23	0.010158	Control Vs Moderate
73	GNG11	3.58	0.01208	Moderate Vs Severe
74	SNORA80A	-3.08	0.012093	Mild Vs Severe
75	SNORA31	-3.3	0.012454	Mild Vs Moderate
76	EMR3	3.71	0.012704	Moderate Vs Severe

77	RNU2-63P	5.83	0.013441	Moderate Vs Severe
78	RNU2-63P	-7.99	0.013635	Control Vs Moderate
79	TRAV26-2	4.13	0.014025	Control Vs Severe
80	SNORD99	-4.4	0.014371	Mild Vs Moderate
81	RNVU1-19	-4.28	0.017484	Mild Vs Moderate
82	RNU5D-1	3.63	0.017608	Moderate Vs Severe
83	GUSBP3	-3.43	0.018055	Moderate Vs Severe
84	SNORA84; MIR3651	-3.23	0.018418	Moderate Vs Severe
85	RNU2-63P	-3.59	0.019558	Mild Vs Moderate
86	MIR3945	-4.44	0.019914	Mild Vs Moderate
87	RNU1-59P	-3.39	0.019921	Mild Vs Moderate
88	GUCY1B3	3.83	0.020658	Mild Vs Severe
89	ITGB3; RP11-290H9.2	3.59	0.022051	Moderate Vs Severe
90	TRAV26-2	5	0.023998	Control Vs Mild
91	SNORA37	-3.16	0.025239	Mild Vs Severe
92	SCARNA5	3.79	0.027708	Control Vs Mild
93	LOC100287834	-4.62	0.027777	Mild Vs Severe
94	RNA5SP199	4.37	0.031178	Control Vs Mild
95	RNU5E-4P	-5.25	0.036353	Control Vs Moderate
96	SNORA33	-3.34	0.038254	Mild Vs Severe
97	GNG11	3.67	0.038262	Control Vs Severe
98	RNVU1-19	-4.51	0.038485	Control Vs Moderate
99	SNORA38B	-3.75	0.044623	Moderate Vs Severe
100	RNU5D-1	-3.89	0.045319	Mild Vs Moderate

*: Gene symbol source was RefSeq database

Table 8S. GSEA significant results for “mild *versus* severe” and “moderate *versus* severe” comparisons
Pathways were ordered by nominal *p*-value.

A. MILD VS SEVERE				
Pathway	Gene Set Size	Enrichment Score (ES)	Normalized ES	NOM p-value
UV_RESPONSE_DN	135	0.376	1.582	0
INFLAMMATORY_RESPONSE	195	0.669	1.213	0
COMPLEMENT	185	0.428	1.387	0.021
ALLOGRAFT_REJECTION	192	0.456	1.478	0.038
UV_RESPONSE_UP	151	0.406	1.333	0.044
APICAL_JUNCTION	193	0.366	1.350	0.046
IL6_JAK_STAT3_SIGNALING	85	0.589	1.251	0.048
KRAS_SIGNALING_UP	188	0.467	1.312	0.049
TGF_BETA_SIGNALING	53	0.436	1.568	0.059
COAGULATION	130	0.469	1.342	0.070
HEME_METABOLISM	188	0.464	1.569	0.084
IL2_STAT5_SIGNALING	193	0.460	1.493	0.090
PI3K_AKT_MTOR_SIGNALING	101	0.339	1.441	0.098
B. MODERATE VS SEVERE				
Pathway	Gene Set Size	Enrichment Score (ES)	Normalized ES	NOM p-value
OXIDATIVE_PHOSPHORYLATION	194	0.502	2.003	0
PEROXISOME	101	0.422	1.686	0
FATTY_ACID_METABOLISM	153	0.432	1.668	0
PROTEIN_SECRETION	92	0.474	1.871	0.018
DNA_REPAIR	134	0.507	1.739	0.018
ADIPOGENESIS	187	0.371	1.666	0.019
HEME_METABOLISM	188	0.476	1.765	0.036
MYC_TARGETS_V2	54	0.611	1.587	0.037
BILE_ACID_METABOLISM	111	0.299	1.250	0.089
XENOBIOTIC_METABOLISM	191	0.360	1.400	0.092

NOTE: The source was HALLMARKS for all pathways.

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