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ORIGINAL ARTICLE

Autoimmunity and Clinical Immunology

Deciphering the role of platelets in severe allergy by an integrative omics approach

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Abstract

Background: Mechanisms causing the onset and perpetuation of inflammation in severe allergic patients remain unknown. Our previous studies suggested that severe allergic inflammation is linked to platelet dysfunction.

Methods: Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) samples were obtained by platelet-apheresis from severe ($n = 7$) and mild ($n = 10$) allergic patients and nonallergic subjects ($n = 9$) to perform platelet lipidomics by liquid chromatography coupled to mass spectrometry (LC–MS) and RNA-seq analysis. Significant metabolites and transcripts were used to identify compromised biological pathways in the severe phenotype. Platelet and inflammation-related proteins were quantified by Luminex.

Results: Platelets from severe allergic patients were characterized by high levels of ceramides, phosphoinositols, phosphocholines, and sphingomyelins. In contrast, they showed a decrease in eicosanoid precursor levels. Biological pathway analysis performed with the significant lipids revealed the alteration of phospholipases, calcium-dependent events, and linolenic metabolism. RNAseq confirmed mRNA overexpression of genes related to platelet activation and arachidonic acid metabolism in the severe phenotypes. Pathway analysis indicated the alteration of NOD, MAPK, TLR, TNF, and IL-17 pathways in the severe phenotype. P-Selectin and IL-17AF proteins were increased in the severe phenotype.

Carmela Pablo-Torres, Elena Izquierdo, Tiak Ju Tan, Mohamed H. Shamji and María M. Escribese equally contributed.

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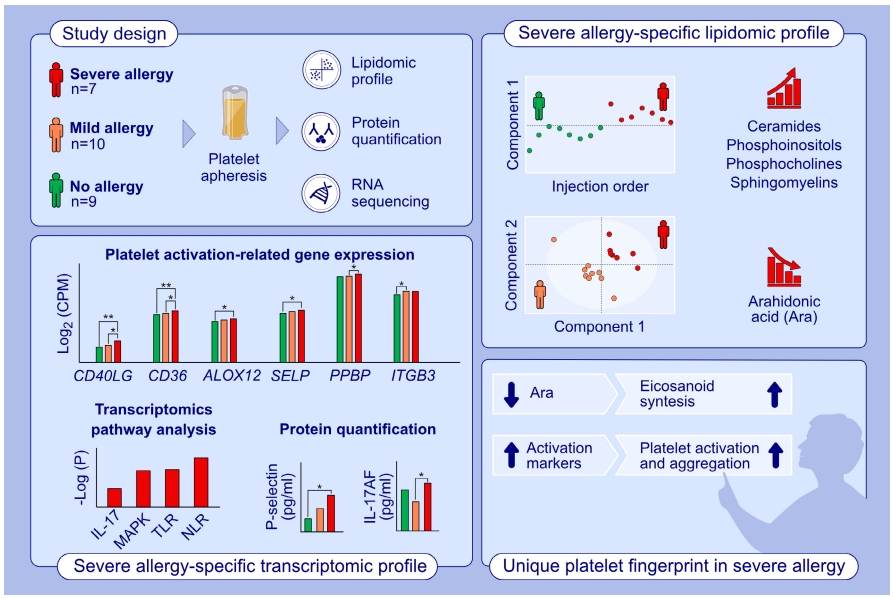
Conclusions: This study demonstrates that platelet lipid, mRNA, and protein content is different according to allergy severity. These findings suggest that platelet load is a potential source of biomarkers and a new chance for therapeutic targets in severe inflammatory pathologies.

KEYWORDS
allergy, lipidomics, metabolomics, platelets, RNAseq



GRAPHICAL ABSTRACT
This study analyzes the lipidomic and transcriptomic profile of platelets in patients with mild and severe allergy. The lipidomic profile of platelets form severe allergic patients shows high levels of ceramides, phosphoinositols, phosphocholines, and sphingomyelins and low levels of arachidonic acid. Transcriptomics reveals a higher expression of platelet activation genes and the alteration of IL-17, MAPK, TLR, and NLR pathways in the severe phenotype, which was validated by P-Selectin and IL-17 AF protein quantification.
Abbreviations: ALOX12, arachidonate 12-lipoxygenase; CD40L, CD40 ligand; CPM, counts per millon; IL, interleukin; ITGB3, integrin subunit beta 3; −Log(P), −Logarithm in base 10 of P value; MAPK, mitogen-activated protein kinase; NLR, NOD-like receptor; PPBP, pro-platelet basic protein; SELP, selectin P; TLR, Toll-like receptor

1 | INTRODUCTION

Allergic diseases are one of the top three conditions demanding a major effort toward prevention and control in the 21st century.[1] In this context, especially severe allergic phenotypes represent a clinical challenge. Severe allergic patients often suffer exacerbations, leading to a chronic inflammatory status that induces irreversible damage on the epithelial barrier and lead to an increase of immune cell infiltrated. Likewise, these patients display systemic alterations in their glucose, sphingolipid, and lysophospholipid metabolism.[2-4] Severe allergic patients generally do not respond to available therapies like high doses of corticosteroids, immunotherapy, or even biological drugs.[3,5] Consequently, patients with a severe phenotype present a poor quality of life, display several comorbidities, and undergo numerous hospital admissions along the years.[6,7] This is not the case for patients with a mild/moderate allergic phenotype, who are able to control the inflammatory response and respond to treatment. Currently, we still do not know the mechanisms underlying the acquisition and maintenance of inflammatory severe phenotypes. Answering this question would contribute to identify novel biomarkers essential for the stratification of patients and to provide novel therapeutic targets for personalized interventions that could prevent the evolution of inflammation to a chronic state.

We have previously indicated that severe allergic inflammation could be associated with the alteration of platelet functions, including activation, adhesion, aggregation and granule secretion.[8] As anucleate cells, platelets can synthesize a limited number of proteins from their preloaded mRNA. Inflammation can alter transcriptional landscape of platelets, as it has been reported for human platelets during sepsis.[9] Several studies pointed out modifications on platelet functions in inflammatory diseases.[10,11] Platelet activation is increased in patients with chronic obstructive pulmonary disease (COPD) during an acute exacerbation, and it is also associated with COVID-19 severity and mortality.[12,13] Moreover, increased levels of platelet-derived mediators have been noted in peripheral blood and bronchoalveolar lavage fluid (BALF) of asthmatic patients, suggesting increased levels of platelet activation.[14]

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Platelet activation is associated with strong metabolic changes, especially in lipid metabolism. In fact, newly generated lipids upon platelet activation can modulate endothelial and immune cells after their release.[15] Previous evidence suggests the importance of platelet lipid signaling in other inflammatory disorders such as sepsis.[15] However, this is an unexplored field in the context of allergy.

A current problem when working with platelets is the large amount of blood needed for obtaining enough platelets. Therefore, for research purposes, platelets are usually obtained from pooled blood samples and isolated by numerous centrifugation steps followed by CD45 depletion.[16] Platelet-apheresis is a technique used for therapeutical purposes that allows the generation of platelet-rich plasma (PRP) from a single donor. This technique grants obtaining a high concentration of platelets without leukocyte or red blood cell contamination,[17] but is not commonly used in basic research, since it is not always available.

Here, we analyze the lipidomic and transcriptomic profile of platelet-apheresis isolated platelets of respiratory allergic patients stratified according to the severity of their phenotype, in order to decipher how platelets contribute to severe allergic inflammation. Understanding platelet biology and their role in the onset of severe inflammatory response will shed light into the mechanisms associated with severe allergic phenotypes and provide novel opportunities for a personalized therapeutic approach.

2 | METHODS

2.1 | Patients

Twenty-six individuals (aged 18–55) were recruited between October 2018 and February 2021 (Table 1). Sample collection from patients was performed out of the pollen season, from October to February, except from M-7 whose PRP was collected in June. The protocol was approved by the Committees of Research and Ethics from the Hospital Universitario Puerta de Hierro Majadahonda (HUPHM), and written informed consent was obtained from all subjects. Nine subjects were nonallergic, proven by Skin Prick Test (SPT), and used as controls. The remaining subjects were allergic patients recruited from HUPHM Allergy Service. Inclusion criteria for allergic patients were clinical history of allergy to aeroallergens proven by SPT. Allergic patients were stratified by severity in mild and severe groups according to GINA (Global Initiative for Asthma) guidelines. Severe patients (GINA Step 5) met at least one of the following criteria: (1) Poor asthma control assessed by ACT (asthma control test) < 20 or ACQ (asthma control questionnaire) > 1.5; (2) Two or more severe exacerbations/ two or more glucocorticosteroid cycles of more than 3 days each (along the previous year); (3) one or more hospitalizations for a severe exacerbation (in the previous year). The rest of patients belonged to GINA steps 3–4 and were included in the mild group. Daily dose of corticosteroid usage for mild and severe patients is shown in Table S7. Patients younger than 18 years old with concomitant inflammatory diseases, cancer, or hematological diseases were excluded.

2.2 | Sample collection and processing

Platelet-apheresis was performed in the apheresis unit (hematology department) of HUPHM. Trima Accel machine (Terumo BCT) was set to obtaining PRP (85 ml) and platelet-poor plasma (PPP) (50 ml) samples using anticoagulant citrate dextrose solution A (ACD-A). Full descriptions of sample collection and characteristics are available in Appendix S1.

2.3 | Metabolomic analysis

PPP and PRP samples (Table S1) were measured using liquid chromatography coupled to mass spectrometry (LC–MS) with a quadrupole-time of flight (Q-TOF) analyzer (Agilent series 6520). Full descriptions of sample preparation, instrumental description, data treatment, and metabolites identification are available in Appendix S1. Physicochemical properties and analytical parameters of identified metabolites are shown in Table S9.

2.4 | Transcriptomic analysis

Platelet RNA was obtained as previously described.[18] Briefly, thawed platelet RNA samples from control, mild, and severe individuals ($n = 3$) were processed for DNA contamination using the DNase1 (Thermofisher), ribo-depleted and then immediately processed for library preparation (Table S1). Full process description is available in Appendix S1.

2.5 | Luminex

A customized panel of six proteins, including P-selectin, interleukin-17AF (IL-17AF), platelet-derived growth factor $\beta\beta$ (PDGF$\beta\beta$), hepatocyte growth factor (HGF), vascular endothelial growth factor A (VEGFA), and MCP1 (monocyte chemoattractant protein-1) from thawed PPP and PRP samples of control ($n = 6$), mild ($n = 6$), and severe ($n = 4$) subjects, was measured using Luminex technology (Thermo Fisher Scientific) (Table S1). Full process description is available in Appendix S1.

2.6 | Statistics

Frequency of categorical patient variables was calculated and analyzed by Fisher's exact test and Chi-squared test. Mean and 95% confidence interval were calculated for continuous variables and

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TABLE 1 Patients' clinical information

Characteristics	Control group ($n = 9$) Mean (95% CI) or Freq (%)	Mild group ($n = 10$)	Severe group ($n = 7$)	p-value
Demographics (allergy service HUPH)				
Gender (male/female)[a]	2 (22.2%)/7 (77.8%)	3 (30%)/7 (70%)	0 (0%)/7 (100%)	.2915
Age[b]	31 (26.34, 35.66)	33.70 (27.61, 39.79)	36.43 (25.61, 47.25)	.5110
Smoking (yes/no)[a]	1 (11.1%)/8 (88.9%)	2 (20%)/8 (80%)	0 (0%)/7 (100%)	.4457
Onset age[c]		15.80 (9.280, 22.32)	17.29 (9.970, 24.60)	.6674
Reactions (RC/RC + AS)[d]		2 (20%)/8 (80%)	0 (0%)/7 (100%)	.4853
SPT (yes/no)[d]				
Olive		10(100%)/0 (0%)	3 (42.86%)/4 (57.14%)	.0147*
Grass		10(100%)/0 (0%)	4 (57.14%)/3 (42.86%)	.0515
Cupressus arizonica		7 (70%)/3 (30%)	6 (85.71%)/1 (14.29%)	.6029
Platanus		6 (60%)/4 (40%)	1 (14.28%)/6 (85.71%)	.134
Cynodon		6 (60%)/4 (40%)	3 (42.86%)/4 (57.14%)	.6372
Weeds		7 (70%)/3 (30%)	2 (28.57%)/5 (71.43%)	.1534
Fraxinus		2 (20%)/8 (80%)	1 (14.28%)/6 (85.71%)	>.9999
Profilin		2 (20%)/8 (80%)	1 (14.28%)/6 (85.71%)	>.9999
Alternaria		2 (20%)/8 (80%)	0 (0%)/7 (100%)	.4853
Dpt		1 (10%)/9 (90%)	4 (57.14%)/3 (42.86%)	.1007
Dfar		2 (20%)/8 (80%)	4 (57.14%)/3 (42.86%)	.1618
Cat		5 (50%)/5 (50%)	3 (42.86%)/4 (57.14%)	>.9999
Dog		1 (10%)/9 (90%)	4 (57.14%)/3 (42.86%)	.1007
FVC[d] (≥80%/<80%)		6 (60%)/0 (0%)	2 (28.57%) /5 (71.43%%)	.021*
FEV1[d] (≥80%/<80%)		6 (60%)/0 (0%)	2 (28.57%) /5 (71.43%%)	.021*
PLTs ($\times 10^9$/L)[b]	238.1 (189.3, 286.98.3)	263.2 (233.6, 292.8)	271.7 (229.1, 314.3)	.2984
WBC ($\times 10^9$/L)[b]	6,3 (5.54, 7.111)	6.955 (5.622, 8.288)	7.057 (5.126, 8.989)	.6724
PRP hemogram (transfusion unit HUPH)				
MPV (fL)[b]	9.12 (8.67, 9.57)	9.22 (8.77, 9.66)	9.21 (8.60, 9.82)	.9237
PLTs ($\times 10^9$/L)[b]	1006 (777, 1235)	1189 (993.7, 1385)	1129 (917.0, 1341)	.4815

Abbreviations: AS, asthma; CI, confidence interval; Dfar, dermatophagoides farinae; Dpt, dermatophagoides pteronyssinus; FEV1, first second of forced respiration; Freq, frequency; FVC, forced vital capacity; MPV, mean platelet volume; PLTs, platelets; RC, rhinoconjunctivitis; SPT, skin prick test; WBC, white blood cells.

* indicates p-value ⟨.05.

[a]Chi-square.

[b]Kruskal-Wallis test.

[c]Mann–Whitney test.

[d]Fisher's exact test.

analyzed with Mann–Whitney U test and Kruskal–Wallis test. Statistical significance was set at 95% level ($p < .05$).

Multivariate analysis was performed using SIMCA P+14.0 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) was used to observe data patterns. Partial least-square discriminant analysis models (PLS-DA) were used to observe differences between clinical groups. The robustness of the models was evaluated by R^2 (explained variance) and Q^2 (capability of prediction) scores. Fold change was calculated for all metabolites in every pairwise comparisons, and those metabolites whose fold change was within the range 0.80–1.20 were excluded for univariant statistics. Univariant statistics to identify potential biomarkers was performed using Matlab R2015a (Mathworks) by nonparametric Mann–Whitney U test and calculating Benjamini–Hochberg p value (PBH) (Table S8). As this is an observational study, statistical significance was set at 95% level ($p < .05$) corresponding with PBH <0.35. This allows to find more potential biomarkers, although validation in further studies is needed to ensure that the resulting biomarkers can properly classify the patients. Significative metabolites obtained by univariant statistics were used for heatmaps and hierarchical clustering, using Euclidean distance measure as clustering parameter with RStudio1.4. IMPaLA version 13 was used for the enrichment pathway analysis.

Protein concentration was calculated interpolating Luminex fluorescence values using a Log5P calibration curve constructed with known concentration standards. Differences in protein concentration between groups were studied with ANOVA after

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performing a normality test. A *p*-value lower than .05 was considered significant.

3 | RESULTS

3.1 | Patient classification

Clinical history of all the subjects was thoroughly analyzed. There were no differences related to sex, age, smoking status, or onset age among the groups ($p > .05$) (Table 1). All severe patients ($n = 7$) presented rhinoconjunctivitis and asthma, as well as most of mild patients (80%). Patients did not show differences regarding their sensitization profile, aside from olive pollen ($p < .05$), in which case all mild patients presented sensitization while only three severe patients were sensitized. Forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1) showed statistically significant differences between mild and severe groups, finding pathological levels (<80%) of these parameters in most severe patients. Whole-blood hemograms showed no differences regarding platelet and white blood cell counts between experimental groups. Hemograms of PRP revealed that platelet counts and mean platelet volume were not different between experimental groups. Statistics of demographic data for each omic approach is shown on Tables S4–S6. Individual data of all the patients are shown in Tables S2 and S3.

3.2 | PRP of severe allergic patients display a particular lipidomic fingerprint

As platelet activation has been associated with changes in lipid metabolism[15], the lipidomic profile of platelets obtained from respiratory allergic patients with different degree of severity was studied using LC–MS. Initially, from 1855 and 767 chemical signals obtained by LC–MS in positive and negative ionization modes, respectively, 210 and 197 complied with the quality criteria. Data quality was assessed by clustering quality control (QC) measurements in a nonsupervised model using PCA (Figure S1). First, we investigated whether the lipidomic profile from PPP and PRP was different. LC–MS data in positive mode from samples of control subjects was examined by a PCA model, which showed a clear clustering of paired PRP and PPP samples (Figure 1A). These results confirmed that platelets display a particular lipid fingerprint.

To clarify whether platelet lipidome could classify patients by inflammatory status, the PRP lipidomic data was studied by a PCA model of the three experimental groups (Figure 1B) and by a PLS-DA comparing two-by-two with the severe group (Figure 1C,D). Although no clustering was observed when comparing the three groups in a PCA, a strong classification was observed when comparisons were conducted against the severe group (Figure 1C,D). The results confirmed that platelets from severe allergic patients displayed a lipidomic profile that differentiates them from nonallergic ($R^2 = 67.1\%$ and $Q^2 = 33.1\%$) and mild ($R^2 = 92.5\%$ and $Q^2 = 20.3\%$) subjects. Next, we identified which particular lipids were the most determinant for the differences observed between groups. Hierarchical clustering showed that severe allergic patients were clustered when comparing them to control and mild subjects, especially in the latter case (Figure 2). However, light clustering was observed when comparing control subjects and mild patients. Therefore, the lipidic profile from platelets of severe patients certainly differs from the other groups.

3.3 | Identification of platelet lipids and biological pathways altered in the severe allergic phenotype

We aimed to identify which particular lipids characterized platelets in each distinct allergic phenotype. For this purpose, we studied lipid abundances in every pair comparison (control vs mild, control vs severe, and mild vs severe), in PRP against the corresponding PPP (Figure 3A). We determined those that were only significantly different in PRP but not in PPP (Figure 3A, Table S8). Only one lipid was altered in platelets when comparing control and mild group. Nonetheless, six lipids were significantly different when comparing the platelet content of the severe patients with either control or mild group (Figure 3A). Abundances of these lipids were plotted in Figure 3B. In the comparison of control vs severe, we identified PC (P-16:0/18:2), PI (18:0/20:3), arachidonic acid (C20:4), PC (16:0/16:0), and Cer (d18:2/23:0) and Cer (d18:2/22:0), all of them increased in the severe phenotype except from C20:4 which showed decreased levels in the severe group (Figure 3B). Also, six lipid alterations exclusive of PRP were identified in mild vs severe comparison: PC (16:0/16:0), Cer (d18:1/16:0), SM (d18:1/24:0), LysoPC (0:0/16:0), LysoPI (20:4/0:0), and LysoPI (18:0/0:0), all of them increased in the severe phenotype (Figure 3B). We observed an increasing trend along severity in the expression of PC (P-16:0/18:2), PI (18:0/20:3), PC (16:0/16:0), Cer (d18:2/23:0), Cer (d18:1/16:0), and SM (d18:1/24:0). In contrast, C20:4 and LysoPC (20:4/0:0) decreased along severity. Of interest, LysoPIs were decreased in the mild group.

Next, we identified which biological pathways were related to the lipid alterations found for each phenotype. The differences in platelet-lipidic content between control and severe group revealed the alteration of phospholipases A2 (PLA2) and C (PLAC), calcium-dependent events, linoleic, linolenic, and arachidonic acid (AA) metabolism (Figure 3C, control vs severe). In contrast, platelet lipidic differences between mild and severe phenotypes showed alterations in SM and Cer metabolism, glutathione redox reactions, transport of PC and PI, and TNF (tumor necrosis factor) signaling pathway (Figure 3C, mild vs severe).

3.4 | Severe allergic patients present a distinct transcriptomic profile

We next questioned whether platelet transcriptome was also altered in the severe group. We performed bulk RNA sequencing on PRP samples ($n = 3$) of the three experimental groups to obtain a global snapshot of the platelet transcriptome. The PCA confirmed the

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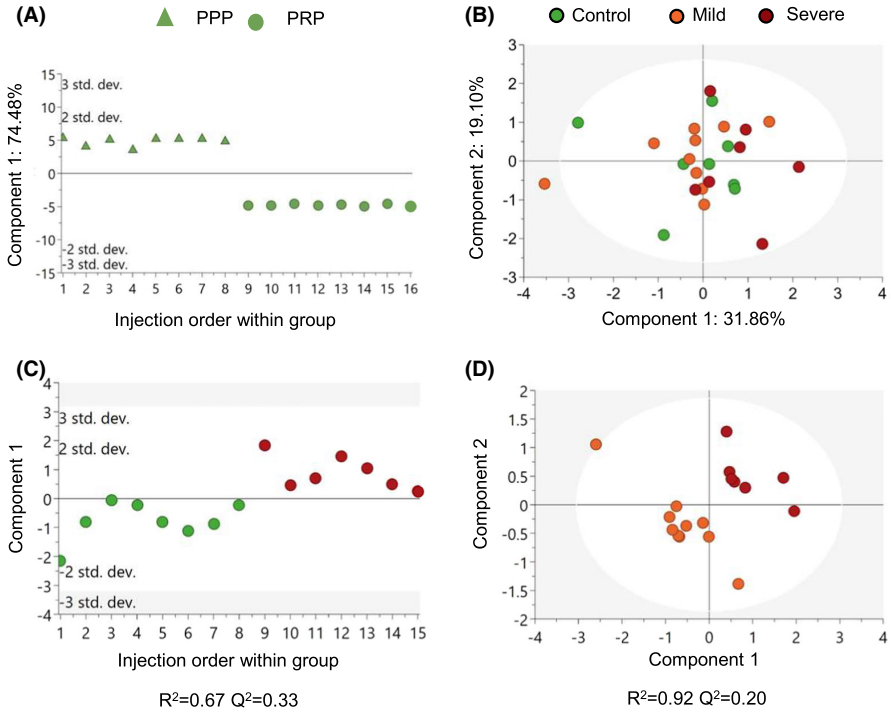

FIGURE 1 PRP from severe allergic patients present a specific lipidomic profile. (A) PCA of LC–MS-positive mode showing differences between PPP (triangles) and PRP (circles) of control subjects ($n = 8$). Data was log transformed and center scaled. *Y* axis represents the percentage of variability explained by the model. *X* axis indicates sample injection order. Sample injection order was C-1, C-4, C-6, C-7, C-2, C-3, C-9 and C-8 for both, PPP and PRP. (B) PCA of LC–MS-positive mode showing differences between Control ($n = 8$), Mild ($n = 10$) and Severe ($n = 7$) subjects in PRP samples. Data was log transformed and center scaled. *X* and *Y* axis indicates the percentage of variability explained by each component. (C) PLS-DA of LC–MS-positive mode showing differences between Control ($n = 8$) vs Severe ($n = 7$) in PRP samples. *X* axis indicates sample injection order in each group. One component was displayed and is indicated by *Y* axis. (D) PLS-DA of LC–MS-positive mode showing differences between Mild ($n = 10$) vs Severe ($n = 7$) in PRP samples. Data was log transformed and center scaled. Two components were displayed and are represented by *X* and *Y* axis respectively.

separation of the severe cluster from the mild and control cluster, indicating a unique fingerprint of the platelet transcriptome of severe patients (Figure 4A). To further explore the specific differences associated to the severe allergic phenotype, pairwise differential gene expression analysis was performed (Table S10). The severe allergic group showed a greater number of differentially expressed genes (DEGs) versus control (815 genes) and vs mild (183 genes) groups, compared to the mild vs control (90 genes) (Figure 4C). Strikingly, a total of 111 genes were common between the pairwise comparisons of severe against mild and control (Figure 4B). Of the top 100 DEGs, patients in the severe group exhibited a distinct transcriptomic signature to that of the control and mild groups as represented in the heatmap (Figure 5). This difference was less pronounced in the mild vs control comparison (Figure 5A). Taken together, these results suggest that the platelets in the severe group are transcriptionally distinct from those of the mild and control groups.

3.5 | Platelets from severe allergic group display inflammatory and activation features

To determine the mechanistic insights and the transcriptional differences in platelets from severe allergic patients, an enrichment analysis pathway was conducted. We identified inflammatory and platelet-related pathways as the most altered in the severe vs control comparison, highlighting genes associated with IL-17, nuclear factor kappa B (NFkB), TNF, and toll-like receptor signalling pathways, (Figure 6A, Table S11). Additionally, severe patients' platelets were differentiated from those form mild and control subjects in cell–cell adhesion related terms such as GAP junction (Figure 6, Table S11). Nonetheless, the transcriptomic profile of mild patients' platelet displayed platelet activation and focal adhesion differences with control platelet group (Figure 6A, Table S11). These results prompt us to specifically compare the mRNA expression levels of genes involved in platelet activation pathways among severe, mild, and control subjects (Figure 6B). Consistent with the lipidomic results, platelets from severe allergic patients showed increased levels of platelet-activation-related genes, such as *SELP* (Selectin P), *PPBP* (Pro-platelet basic protein), *CD40LG* (CD40 Ligand), and *CD36* and platelet-aggregation-related genes such as ITGB3 (Integrin beta chain beta 3). Of interest, *ALOX12* (arachidonate 12-lipoxygenase), which codifies for a lipoxygenase that participates in AA metabolism, was also increased in the severe group. In addition, an enhancement in P-selectin and IL17-AF protein levels was detected in severe patients' platelets (Figure 6C), while the rest of the quantified proteins did not show significant differences among groups (Figure S2). These results suggest that additionally to the above-mentioned lipidomic fingerprint, platelets

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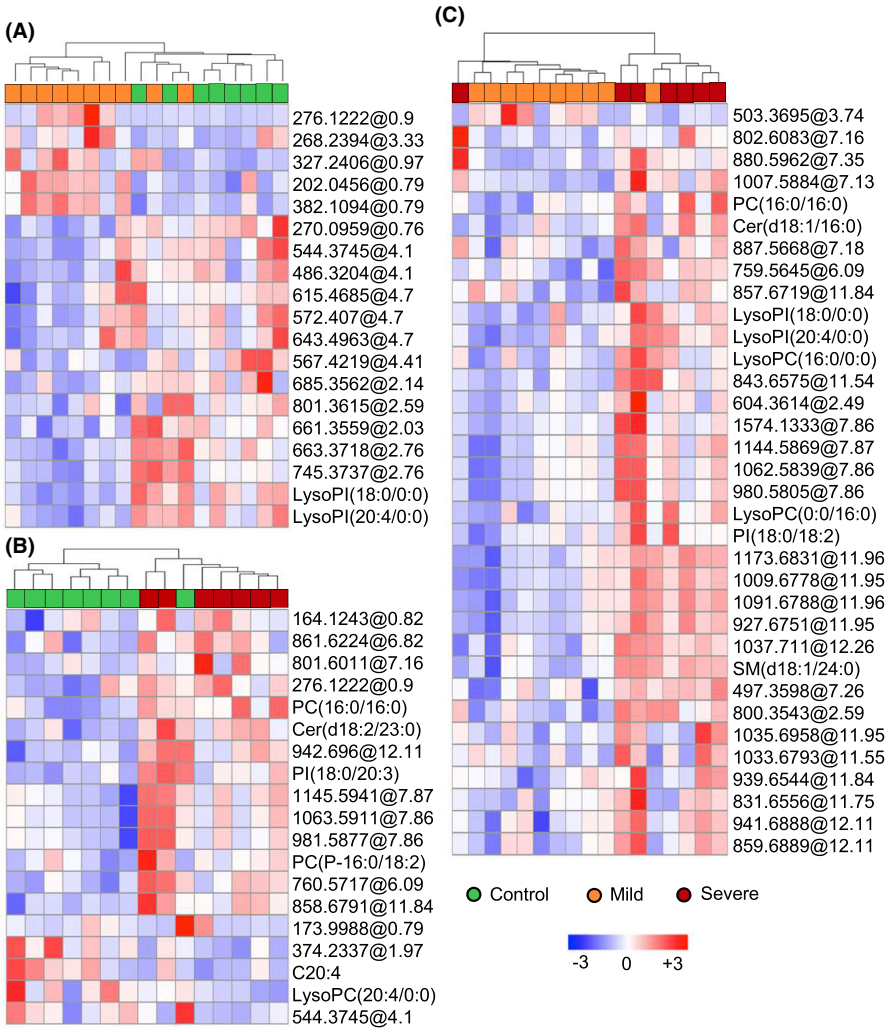

FIGURE 2 Metabolites differentially detected in PRP clusterize severe allergic patients. Hierarchical clustering was performed with the statistically significant lipids (Mann–Whitney *U* test *p* value <.05) between (A) Control vs Mild, (B) Control vs Severe and (C) Mild vs Severe. Each row represents a single metabolite; each column represents an individual PRP sample. Red bands indicate a higher expression level, and blue bands indicate a lower expression level. Unknown features (metabolites without annotation) are represented by "Mass@Retention Time." Detailed information about identified metabolites is available in Tables S8 and S9.

contain specific mRNA transcripts and proteins associated with the severity grade in allergic patients. Altogether, our data undelight the proinflammatory potential of platelets in patients with more severe phenotype.

4 | DISCUSSION

Severe allergy is an heterogeneous and challenging to treat condition with a resilient impact on patient quality life and a high cost to healthcare systems.[3,19,20] Severe allergic patients are commonly not controlled with any combination of the available treatments, display several comorbidities, and, consequently, have a poor quality of live.[2] We believe that a better understanding of the molecular and cellular processes taking place in these patients will provide relevant information for their appropriate stratification and therefore for the design of novel personalized interventions.

Here, we identify a novel mechanism associated with severe allergic phenotypes. We demonstrate that platelets are a source of proinflammatory mediators that differentiate severe from mild allergic patients and that reveal both new immune-regulatory mechanisms and potential novel biomarkers.

In a previous work based on multiomic approaches from peripheral-blood mononuclear cells (PBMC) samples, we found evidence that platelet functions are altered on severe allergic inflammation.[8] In fact, the role of platelets in inflammatory response was also previously described in different disorders.[22-24] In the present study, we have specifically investigated the transcriptomic and lipidomic load of platelets obtained from allergic patients with different grades of severity, and we have obtained a differential fingerprint that shed light in their potential role in the regulation of the allergic inflammatory response.

Allergic patients' severity was assessed using GINA guidelines. Concretely, severe phenotypes belonged to GINA step 5, meaning that patients present exacerbations and/or hospitalizations. Mild patients were classified according to GINA guidelines as step 3–4, with controlled symptoms mainly due to corticosteroids.

Woking with platelets is a challenging task. Cell numbers are usually too low for experimental procedures and activation is a common issue. This explains why platelets experimental approaches usually imply sample pooling and centrifugation protocols as the election of choice for platelet isolation.[16,25] As methodological earlier, we use platelet-apheresis for platelet isolation, a technique that allows the generation of large amounts of PRP per donor with no leukocyte or

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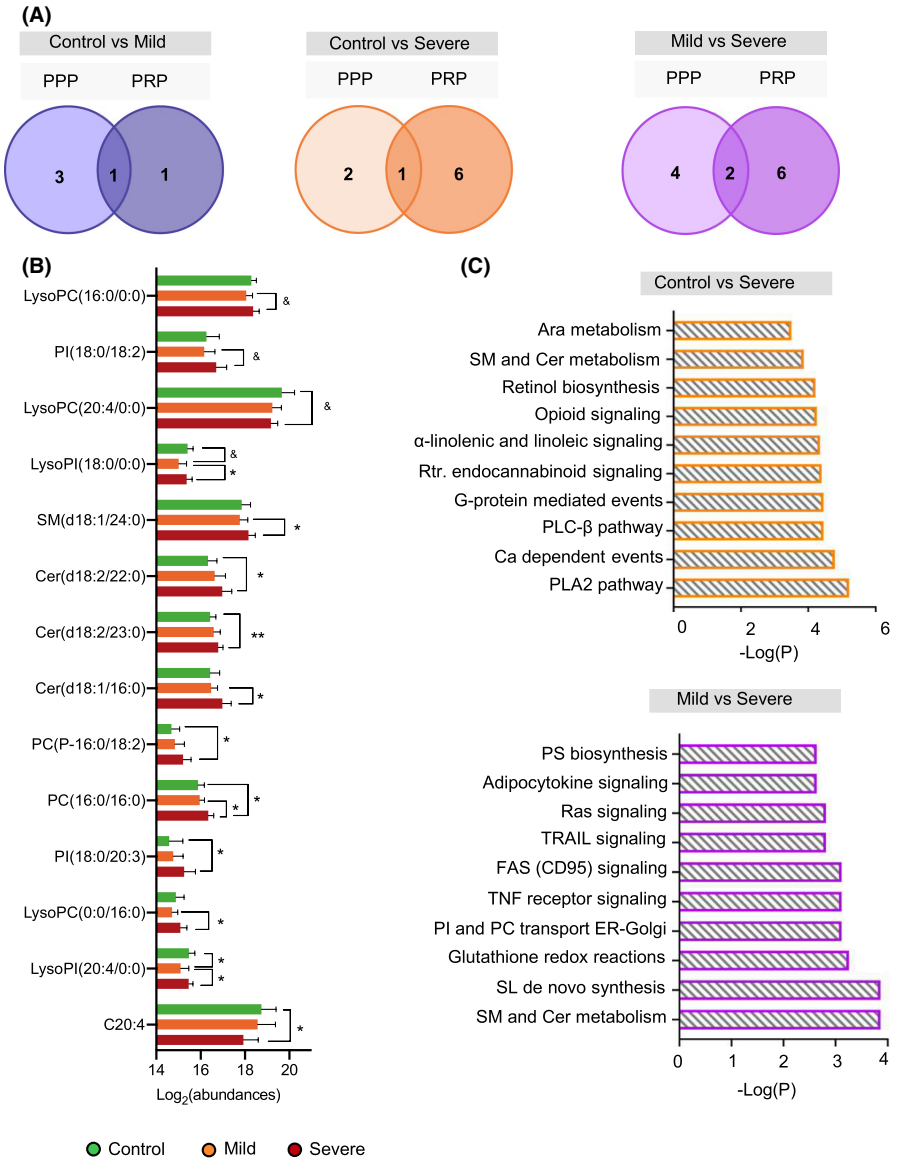

FIGURE 3 Severe patients' platelets have a differential lipidic load. (A) Venn diagrams representing significant differences in PPP (left circles) and PRP (right circles) for every comparison: Control vs Mild (left), Control vs Severe (middle) and Mild vs Severe (right). Intersections shows metabolites that are significantly different in both matrixes. (B) Bar plot representation of the abundance of significant metabolites detected. '&' is used for indicating differences found in PRP and also in PPP. '*' is used for indicating differences found in PRP but not in PPP. Error bars cover the interquartile range. **Mann–Whitney *U* test $p < .005$, * or & Mann–Whitney *U* test $p < .05$. (C) Most differentially detected ($p < .05$) metabolic pathways using Impala Software in which statistically significant metabolites from PRP of Control vs Severe and Mild vs Severe comparisons are involved. Abbreviations: Ara, arachidonic acid; Ca, calcium; Cer, ceramide; LysoPC, lysophosphatidylcholine; LysoPI, lysophosphatidylinositol; PC, phosphatidylcholine; PI, phosphatidylinositol; PL, phospholipase; PS, phosphatidylserine; Rtr, retrograde; SL, sphingolipid; SM, sphingomyelin; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

red blood cell contamination.[18] Therefore, we were able to obtain high yield and pure platelet samples. In fact, this technique is usually used for clinical settings but not for research, since it requires qualified personnel and infrastructure in a hospital.[26] Moreover, this approach generates both types of samples, PRP and PPP. As we demonstrate here, the comparison between PPP and PRP allows to detect which metabolic/transcriptomic modifications are due to specific platelet content, considering no platelet contribution in PPP. As we have recently shown, PRP samples contained quantifiably levels of platelet-related mRNA and proteins, which were lower or undetectable on the PPP.[18]

Interestingly, our results do not demonstrate significant differences regarding platelet hematological parameters, such as plateletcrit, platelet mean value, and platelet counts, between clinical groups enrolled in the study. Therefore, the alterations found could only be attributed to a differential platelet content of every phenotype. Contrary to other reports that show changes in platelet counts are associated with inflammatory status.[27,28]

This study shows that platelets from severe allergic patients presented a different lipidomic and transcriptomic profile than mild allergic patients and nonallergic subjects. This is in line with previous reports from the group where, by using different models, we described specific severe allergic features like: a unique pattern of oral epithelial remodeling,[4,21] and a specific metabolic, transcriptomic,[8] and proteomic[3] signature associated with severity.

Here, we showed that platelets from severe allergic patients contain altered levels of proinflammatory lipids such as sphingolipids (ceramides (Cer) and sphingomyelins (SM)), arachidonic acid (AA), and lysophospholipids (LPC). These lipids have been reported to be involved in the allergic inflammatory response.[29-31] In this work, we have detected an increase in Cer and SM in platelets of severe allergic patients. Cer and SM belong to the family of sphingolipids, and their importance in allergic diseases has been widely studied.[29] It is worth mentioning that we and others have previously shown elevated sphingolipid levels in patients' sera obtained from diverse models of allergic inflammation.[3,8,32] Cer levels have been correlated

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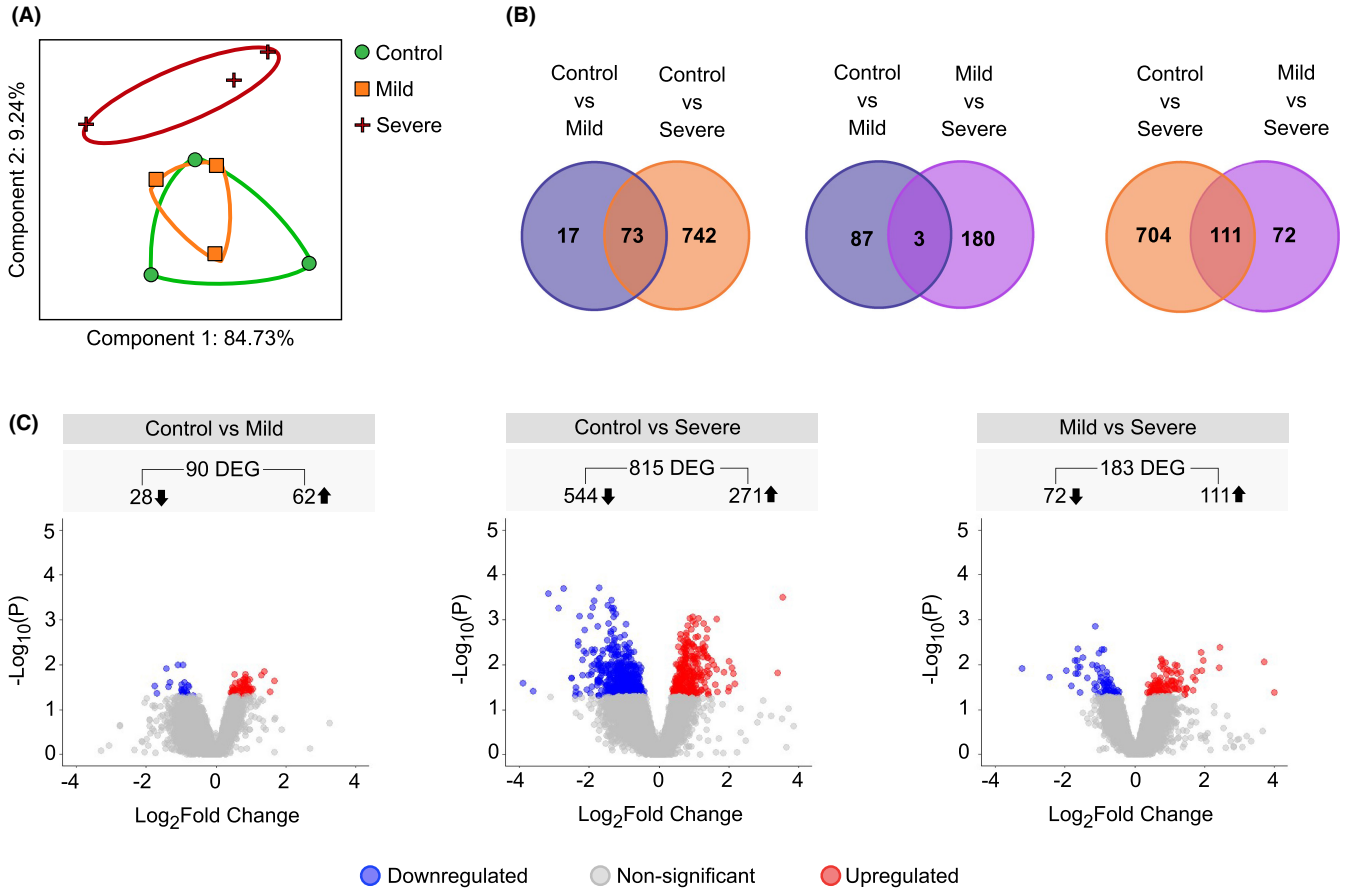

FIGURE 4 Distinct transcriptional signatures of platelets in severe allergics compared to mild and non-allergic individuals. (A) Principal component (PCA) analysis of transcriptome libraries obtained from severe, mild and control groups. (B) Venn diagram depicting the number of unique and intersecting genes from pairwise differential gene analysis. (C) Volcano plots depicting upregulated (Log_2 Fold Change >0, red) and downregulated (Log_2 Fold Change <0, blue) genes from each pairwise differential gene analysis. Genes are considered differentially expressed with an unadjusted $p < .05$.

to asthma severity, and James et al. reported higher levels of Cer BALF from patients suffering from severe asthma, compared with mild asthmatic patients and healthy controls.[33] In addition, an untargeted metabolomics analysis of serum from healthy individuals and asthmatic patients also found that increased levels of Cer and SM positively correlated with asthma severity.[34] Interestingly, a causal relationship between plasma levels of different subclasses of Cer, including Cer (d18:1/16:0), and the onset of COVID-19 respiratory distress symptoms has been inferred.[35] Concordantly, we have detected this specific Cer subclass increased in platelets from severe allergic patients. It is known that Cer is implicated in platelet activation and endothelial dysfunction.[36] Besides, inflammatory cytokines, such as interferon-γ, TNF-α, and interleukin-1ß, stimulate Cer synthesis.[36] Altogether, these data suggest that the chronic inflammatory condition present in severe allergic patients could enhance Cer synthesis by platelets contributing to the maintenance of an inflammatory status.

Moreover, previous studies demonstrated that Cer can interact directly with PLA2, stimulating AA realease.[37] In addition, our results reveal the implication of the PLA2 and PLC pathways and AA metabolism, all of the established processes associated with platelet activation lead to eicosanoid release. Surprisingly, platelets of severe allergic patients presented lower levels of AA. Considering that AA is the precursor for eicosanoids synthesis, these reduced levels suggest an enhanced synthesis of eicosanoids,[38] which are lipid-based signaling molecules in both innate and adaptive immune responses.[39,40] Eicosanoids are known to amplify type 2 immunity by recruitment and activation of eosinophils, Th2 cells, ILC2, monocytes, DCs, and MCs.[40] As they are well-known triggers of the proinflammatory responses, eicosanoid production by patients' platelets could also play a role in severe allergic inflammation. In support of a stronger supply of eicosanoids by severe patients' platelets, our data showed that these platelets are loaded with higher mRNA levels of the *ALOX12* gene, which encodes the lipoxygenase ALOX12. This enzyme generates bioactive lipid mediators, including eicosanoids, and is involved in platelet aggregation and TNF-α, MAPK, and NFkB signaling pathways.[41,42] Since our data also point to significant alterations of the above-mentioned signaling pathways in severe patient's platelets, it is tempting to hypothesize that elevated levels of platelet-*ALOX12* lead to a rise in eicosanoids that subsequently boost inflammation on severe phenotypes. Likewise, we show

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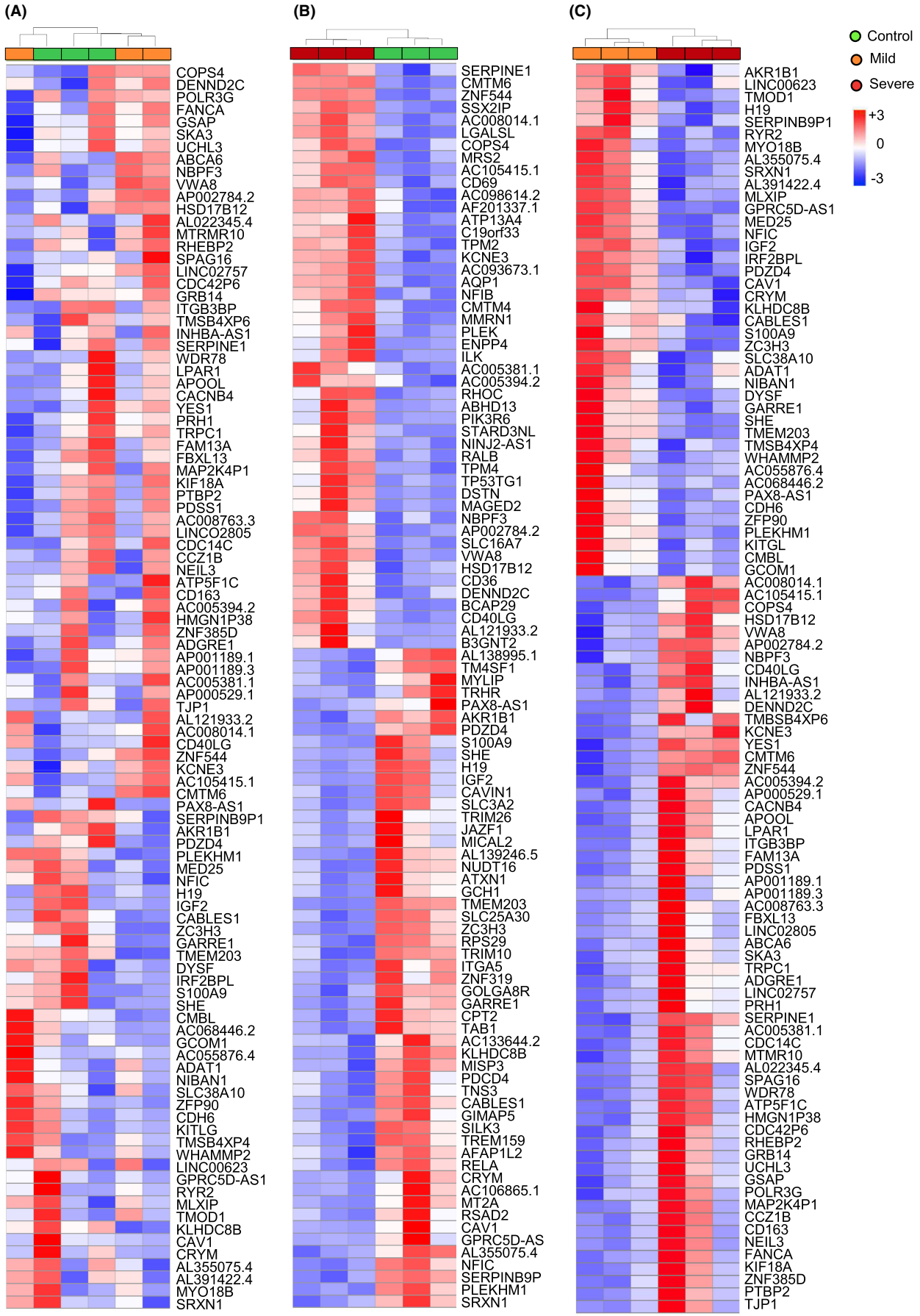

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FIGURE 5 Severe group exhibit a distinct transcriptomic signature to that of the control and mild groups. Heatmaps of the top 100 DEGs ranked by *p*-value from (A) Control vs Mild, (B) Control vs Severe and (C) Mild vs Severe. Hierarchal clustering was performed using the Euclidean distance. The Gene Specific Analysis tool was employed to identify the differentially expressed genes using Partek Flow. Each row represents a single transcript; each column represents an individual PRP sample. Red bands indicate a higher expression level, and blue bands indicate a lower expression level.

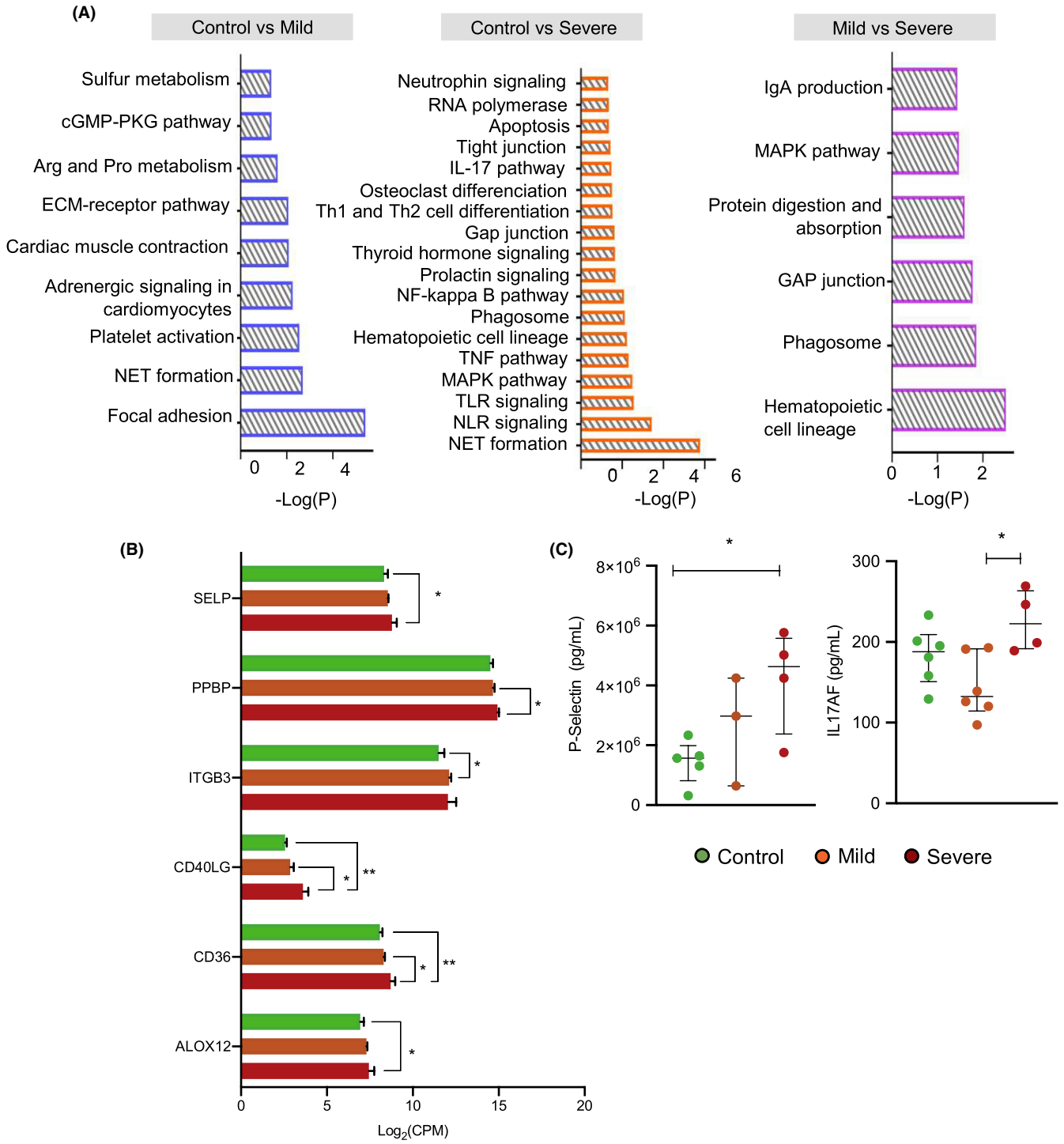

FIGURE 6 Platelets from severe allergic patients present increased expression of platelet activation related transcripts and proteins. (A) Most relevant differentially detected ($p < .05$) biological pathways in which statistically significant metabolites from PRP of Control vs Mild, Control vs Severe and Mild vs Severe comparisons are involved. (B) Bar plot representation of counts per million (CPM) of significant transcripts (T-Student test $p < .05$) detected. Error bars cover the interquartile range. (C) IL-17 and P-Selectin concentration measured by Luminex in PRP samples from Control, Mild and Severe subjects (ANOVA $p < .05$). Abbreviation: Arg, arginine; cGMP, cyclic GMP; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; NET, neutrophil extracellular traps; NLR, NOD-like receptor; PKG, protein kinase G; Pro, proline; TLR, toll-like receptor.

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that platelets obtained from severe patients contain higher protein levels of IL17A, a proinflammatory cytokine detected in severe asthma phenotypes.[43]

Another important lipidomic modification found in platelets from severe allergic phenotypes was the increase of PC (16:0/16:0) and LPC 16:0. We must emphasized that LPC 16:0 was also found to be augmented in PPP of severe subjects, indicating a high systemic content of this molecule in these patients. Similarly, we and others have previously described higher levels of LPC 16:0 in serum samples from uncontrolled asthmatic patients[3] and in BALF of asthmatic patients.[44] In addition, previous reports support that LPC16:0 exert proinflammatory activities, such as eosinophil adhesion[45] and monocyte IL1- β secretion.[46] Thus, the increase of LPC 16:0 and its precursor PC (16:0/16:0) in the PRP of severe allergic patients sustain a role for platelets in the maintenance of chronic inflammatory injury present in severe allergic phenotypes.

Platelets are able to release their content upon activation. When platelets are activated, P-selectin is immediately translocated to the plasma membrane where it acts as a receptor or ligand for its counterpart expressed on the surface of other immune cells (PSGL-1), vital for the initiation of the recruitment of these cells to the site of interest. Our results showed that platelets from severe allergic patients contain increased levels of P-selectin (mRNA and protein), as well as other transcripts related to platelet activation and aggregation (*PPBP*, *CD40*, *CD36*, and *ITGB3*), indicating that platelets are highly activated in the severe group, as has been observed in other inflammatory diseases.[21]

Our data demonstrate that platelets from severe allergic patients are a source of inflammatory mediators that present markers of an enhanced activation state, supporting their role as key players in the pathophysiology of severe allergy inflammation. This observation points out the potential role of platelets for finding novel biomarkers and therapeutic targets for severe allergic patients.

AUTHOR CONTRIBUTIONS

MME was the PI and together with MHS, DB, CB, EI, AV, and CG-C designed and supervised the research. MMR-M recruited and stratified patients and JLB-C supervised sample obtention and quality. LM-B, JS-S, CG-C, EI, and CP-T obtained and processed the samples. CP-T, DO, and AV performed the metabolomics analysis and data treatment. TJT and JAL performed the transcriptomic 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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CONFLICT OF INTEREST

All authors have read and approved the manuscript. Any potential conflicts of interests are listed here: DB reports consulting fees from ALK A/S. CG-C reports funding "Atracción de talento investigador" from Community of Madrid, Spain (2017–2020). MHS reports grants through institution from Regeneron, Merck, ANGANY Inc, Immune Tolerance Network and Allergy Therapeutics, and personal fees from Allergopharma. MME reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from Stallergenes Greer and Diater.

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