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ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Understanding uncontrolled severe allergic asthma by integration of omic and clinical data

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Abstract

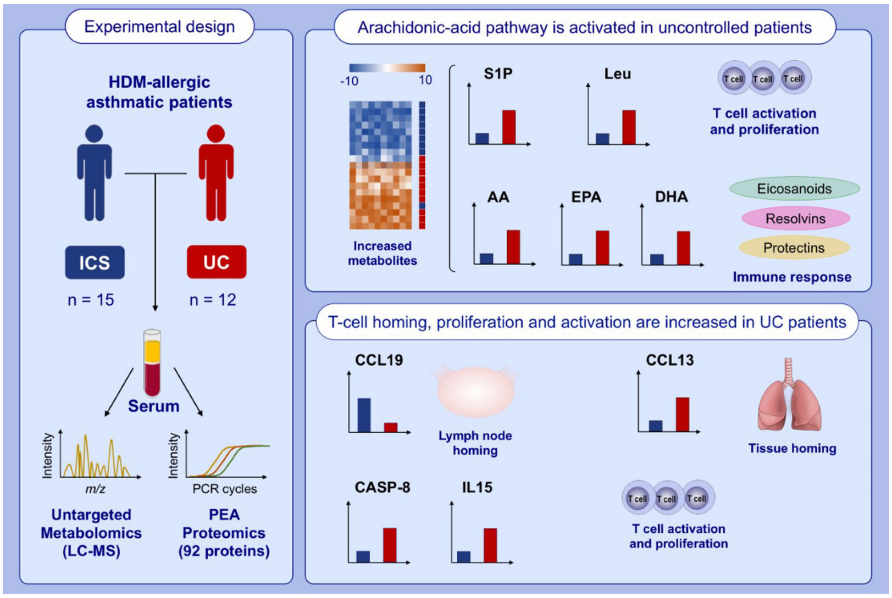
Background: Asthma is a complex, multifactorial disease often linked with sensitization to house dust mites (HDM). There is a subset of patients that does not respond to available treatments, who present a higher number of exacerbations and a worse quality of life. To understand the mechanisms of poor asthma control and disease severity, we aim to elucidate the metabolic and immunologic routes underlying this specific phenotype and the associated clinical features.

Methods: Eighty-seven patients with a clinical history of asthma were recruited and stratified in 4 groups according to their response to treatment: corticosteroid-controlled (ICS), immunotherapy-controlled (IT), biologicals-controlled (BIO) or uncontrolled (UC). Serum samples were analysed by metabolomics and proteomics; and classifiers were built using machine-learning algorithms.

Results: Metabolomic analysis showed that ICS and UC groups cluster separately from one another and display the highest number of significantly different metabolites among all comparisons. Metabolite identification and pathway enrichment analysis highlighted increased levels of lysophospholipids related to inflammatory pathways in the UC patients. Likewise, 8 proteins were either upregulated (CCL13, ARG1, IL15 and TNFRSF12A) or downregulated (sCD4, CCL19 and IFNγ) in UC patients compared to ICS, suggesting a significant activation of T cells in these patients. Finally, the machine-learning model built including metabolomic and clinical data was able to classify the patients with an 87.5% accuracy.

Conclusions: UC patients display a unique fingerprint characterized by inflammatory-related metabolites and proteins, suggesting a pro-inflammatory environment. Moreover, the integration of clinical and experimental data led to a deeper understanding of the mechanisms underlying UC phenotype.

KEYWORDS
allergy, asthma, machine learning, metabolomics, proteomics



GRAPHICAL ABSTRACT
Severe uncontrolled HDM-allergic asthma (UC) displays an increased T-cell activation and proliferation (IL-15, CASP-8, S1P, Leu) and an increased T-cell tissue recruitment (CCL13) compared to corticosteroid-controlled HDM-allergic asthma (ICS). UC shows an exacerbated inflammatory response with increased levels of inflammatory mediators (AA, EPA, DHA, ...). Integration of clinical and metabolomic data is the best strategy to stratify patients by severity.
Abbreviations: AA, arachidonic acid; CASP-8, caspase-8; CCL13, chemokine (C-C motif) ligand 13; CCL19, chemokine (C-C motif) ligand 19; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDM, house dust mites; ICS, inhaled corticosteroid-controlled HDM-allergic asthma; IL-15, interleukin-15; LC-MS, liquid chromatography coupled to mass spectrometry; Leu, leukine; PEA, proximity extension assay; S1P, sphingosine-1-phosphate; UC, uncontrolled HDM-allergic asthma

1 | INTRODUCTION

Asthma is a heterogeneous disease characterized by chronic airway inflammation and a clinical history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation.[1]

Asthma can have multiple phenotypes and endotypes, depending on the clinical features, histopathology, genetics or response to treatment, along with other characteristics.[2] We can distinguish between allergic, intrinsic, neutrophilic, aspirin-intolerant and extensive remodelling asthma,[3] among others. Patients with allergic asthma are usually sensitized to house dust mites (HDM). In fact, IgE titres to HDM correlate with exacerbations in asthma.[4]

HDM are the most relevant inducers of allergic diseases worldwide.[5] HDM allergens include cysteine-proteases (Der p 1, Blo t 1) and chitin-associated proteins (Der p 23), involved in epithelial disruption and remodelling; proteins able to activate TLR4 (Der p 2, Eur m 2), and lipid-binding proteins (Der p 5, Der p 7 and Der p 21), linked with the establishment of the Th2 response.[5] The Canary Islands in Spain is one of the regions in Europe with higher mite exposure levels and sensitization prevalence to HDM due to its almost tropical climate of high humidity and warm temperatures.[6,7] These features are common with other high-exposure HDM areas like Singapore or New Zealand.[5] Additionally, the exposome in the Canary Islands has been deeply studied[8]; and it is very stable along the island, which makes it a perfect model to minimize variability in the study.

Due to the complexity of asthma, effective treatment is also a challenge. The GINA guideline sets a step-by-step approach, in which treatment with increasing doses and add-on medications are given to patients. For allergic asthma, therapeutic strategies include corticosteroids, bronchodilators, immunotherapy and biological drugs (*i.e.*, anti-IgE or anti-IL5).[1]

Classification of asthmatic allergic patients is a difficult task. Several endotypes and phenotypes have been described. According to their response to treatment, patients have severe asthma if they

either needed treatment from steps 4–5 of the GINA,[1] or 5–6 of the GEMA[9] guidelines (which includes inhaled corticosteroids coupled to long term acting beta-agonists (inhaled CS/LABA) in high doses + antileukotrienes (LTRA) + theophylline (T)) or had to take systemic corticosteroids to achieve control in the previous year.

However, despite having received the treatment previously mentioned, around 3%–10% of patients display severe uncontrolled asthma. These patients often present comorbidities such as obesity, nasal polyposis, allergic rhinitis or food allergy; resulting in a worse quality of life and higher mortality rates.[1,10]

Particularly, in the Canary Islands several studies report frequent respiratory problems in children and high rates of asthma prevalence (69.6%). Moreover, 16% of the asthmatic patients have severe uncontrolled asthma,[11] which is similar to other European countries, such as the United Kingdom where it is close to 18%.[12]

Regardless of this significant prevalence, there is no available effective treatment for these patients. Their treatment options rely on the development of new personalized medicine strategies.

In this study, all the patients were included by the Allergy Service at Hospital Universitario de Gran Canaria Dr Negrin, a group with deep knowledge on asthma management that works in an area with high HDM exposure and with a well-known exposome, resulting in a very homogenous cohort of asthmatic patients stratified according to their response to treatment. This is a unique clinical model to study a complex disease such as allergic asthma.

Here, we used untargeted metabolomics and a bioinformatic analysis, in addition to a proteomics approach, to generate a specific fingerprint associated with uncontrolled allergic asthma that conforms a set of biomarkers with a potential use to develop the personalized intervention strategies that these patients need.

2 | METHODS

2.1 | Patients

Patient recruitment was conducted within one full year. Eighty-seven patients with a clinical history of asthma, classified by the GINA guideline[1] and with a positive skin-prick test to HDM were enrolled in the Allergy Department of Hospital Universitario de Gran Canaria Dr Negrín (Las Palmas de Gran Canaria, Spain).

Recruited patients were characterized by a very complete clinical history, since they were monitored for at least 5 years prior to the study. Detailed information about patient sensitization profiling and clinical characteristics can be found in Supplementary. All patients signed informed consent and the study was approved by the Ethics Committee of the hospital on the 4th/February/2016 (code: 160009). Serum samples were obtained as described in Supplementary.

Clinical characteristics were compared between the 4 groups. For continuous variables, Shapiro-Wilk test was applied to assess normality of the data. Age and BMI passed the normality test, so we performed ANOVA test with Tukey's post hoc. Onset age and total IgE, however, did not pass the Shapiro-Wilk test, and were therefore analysed using Kruskal-Wallis with Dunn's post hoc. For categorical variables, chi-square analysis was performed, and for groups with data lower than 5, Fisher's exact was used.

2.2 | Metabolomic analysis

Serum samples were prepared and measured in batches, in a randomized order using a liquid chromatography coupled to mass spectrometry (LC-MS) with a quadrupole-time of flight (Q-TOF) analyser (Agilent series 6520). The experiment, as previously described,[13] was measured in electrospray ionization in positive and negative modes (ESI+ and ESI−, respectively). A quality control (QC) sample was prepared by mixing equal volumes of a representative set of samples and was analysed throughout the analyses to ensure instrumental reproducibility. Metabolite annotation was performed using CEU Mass Mediator 3.0 online data base[14] and confirmed through tandem mass experiments (MS/MS) with a fragmentation energy of 20 eV. Full description of sample preparation, instrumental characteristics, data quality, data treatment, statistical analysis and metabolite annotation are stated in Supplementary.

2.3 | Proteomic analysis

Serum proteins were analysed by Proximity Extension Assay (PEA) (Olink®, Uppsala). PEA is based on the probes linked to paired antibodies recognizing the same protein. Upon the antibodies binding to the target, the primers hybridize and create a PCR product, which is then amplified as the read-out, giving the Normalized Protein Expression (NPX) as result. For this assay, 10 µl of serum of corticosteroid-controlled (ICS) (n = 15) and uncontrolled (UC) patients (n = 11) were analysed by Olink. Therefore, proteomic analysis was not performed in all samples. We used Target 96 Immuno-Oncology panel (95311, Olink®, Uppsala) consisting in 92 proteins involved in processes such as promotion and inhibition of tumour immunity, chemotaxis, vascular & tissue remodelling, apoptosis & cell killing and metabolism & autophagy. Detailed information about quality control, data treatment and statistical analysis can be found in Supplementary.

2.4 | Machine-learning model construction

To gather novel insights about the molecular mechanisms involved in the UC allergic asthma phenotype, we employed multi-omic data analysis. The integration and analysis of omics and clinical data with machine-learning (ML) methods offer novel techniques enabling the discovery of new biomarkers.[15] After data pre-processing and down sampling, clinical (50 variables after the exlusion of medication) and metabolomic (86 identified significant metabolites) data were mixed

to create a ML predictor that could classify ICS and UC patients in separate groups. For the ML model, *caret*, *mlbench*, *sjmisc* and *class* R packages were used for k-Nearest Neighbour (k-NN) algorithm. Proteomic data were not included in this analysis because the samples used for metabolomics and proteomics were not always the same. Full description of the data treatment steps, training and testing can be found in Supplementary.

3 | RESULTS

3.1 | Patient classification

Patients were classified in 4 groups according to the medication that controlled their symptoms: (1) Mild asthma patients controlled with inhaled or topic corticosteroids, and never with systemic corticosteroids (ICS, $n = 15$); (2) mild-moderate asthma controlled with immunotherapy (IT, $n = 44$); (3) moderate-severe asthma patients controlled with omalizumab (anti-IgE) (BIO, $n = 16$); and (4) severe asthma patients that do not respond to any treatment, or uncontrolled patients (UC, $n = 12$). More information about these groups can be found in Supplementary.

Moreover, their clinical history was thoroughly analysed. There were no differences related to sex, ethnicity, BMI, smoking status, total IgE levels and type of HDM sensitization among the groups ($p > 0.05$) (Table 1). However, age was found to be significantly higher in the UC group than in the ICS.

Looking at the medication, patients from the ICS group were treated with antihistamine (AH) and topical corticosteroids (Topic CS), and most of them (93%) administered inhaled CS/LABA. Three of these patients were prescribed LTRA and/or short-effect bronchodilators (SABA). IT patients had a medication pattern like that of ICS patients, although only about a third of them were taking inhaled CS/LABA, while most of them (75%) were prescribed SABA. BIO patients, on the other hand, had more in common with the UC, although the percentage of patients taking topic CS doubled that of UC patients taking this drug (68.8%). The exact treatment and sensitizations of each patient are shown in Table S1. Regarding the UC, all patients were taking both inhaled CS/LABA and SABA, and most of them (75%) were taking LTRA as well. 50% of these

TABLE 1 Clinical information of the patients

	ICS	IT	BIO	UC
N	15	44	16	12
Age (years)	37.2 ± 2.3	37.3 ± 1.5†	43.3 ± 2.4	48.5 ± 3.6*
Onset Age (years)	15.8 ± 2.9	16.1 ± 1.7	12.1 ± 1.8	11.9 ± 2.7
Gender (%F/%M)	66.7/33.3	77.3/22.7	87.5/12.5	58.3/41.7
BMI	26.7 ± 1.4	26.0 ± 0.6	26.8 ± 1.1	28.0 ± 1.2
Smoker (%)	13.3	0	6.3	0
Ex-smoker (%)	6.7	2.3	6.3	25
Total IgE (U)	483.7 ± 164.7	396.2 ± 64.6	503.9 ± 161.5	536.3 ± 181.7
AC (%)	0	0†	43.8*	50*
AH (%)	100	90.9†	37.5*	41.7*
LTRA (%)	13.3	11.4†	75*	75*
Inhaled CS (%)	0	2,3	0	0
Inhaled CS/LABA (%)	93.3	34.1*,†	100	100
Oral CS (%)	0	0	0	16.7
Topic CS (%)	100	97.7†	68.8*	33.3*
SABA (%)	20	75*	93.8*	100*
T (%)	0	0	0	8.3
NSAID-HS (%)	13.3	6.8	6.3	16.7
D. pteronyssinus (%)	93.3	97.7	93.8	91.7
D. farinae (%)	93.3	93.2	87.5	91.7
L. destructor (%)	66.7	50	43.8	58.3
B. tropicalis (%)	86.7	77.3	81.3	66.7
A. siro (%)	26.7	20.5	0	8.3
T. putrescentiae (%)	53.3	45.5	56.3	58.3

Abbreviations: AC, anticholinergic; AH, antihistaminic; BMI, Body mass index; CS, corticosteroids; Inhaled CS/LABA, inhaled corticosteroids combined with Long-acting beta-adrenoceptor agonist; LTRA, antileukotriene; NSAID-HS, NSAID-hypersensitivity; SABA, short-acting beta-adrenoceptor agonist; T, theophylline; U, ISAC units.

*$p < .05$ against ICS. †$p < .05$ against UC.

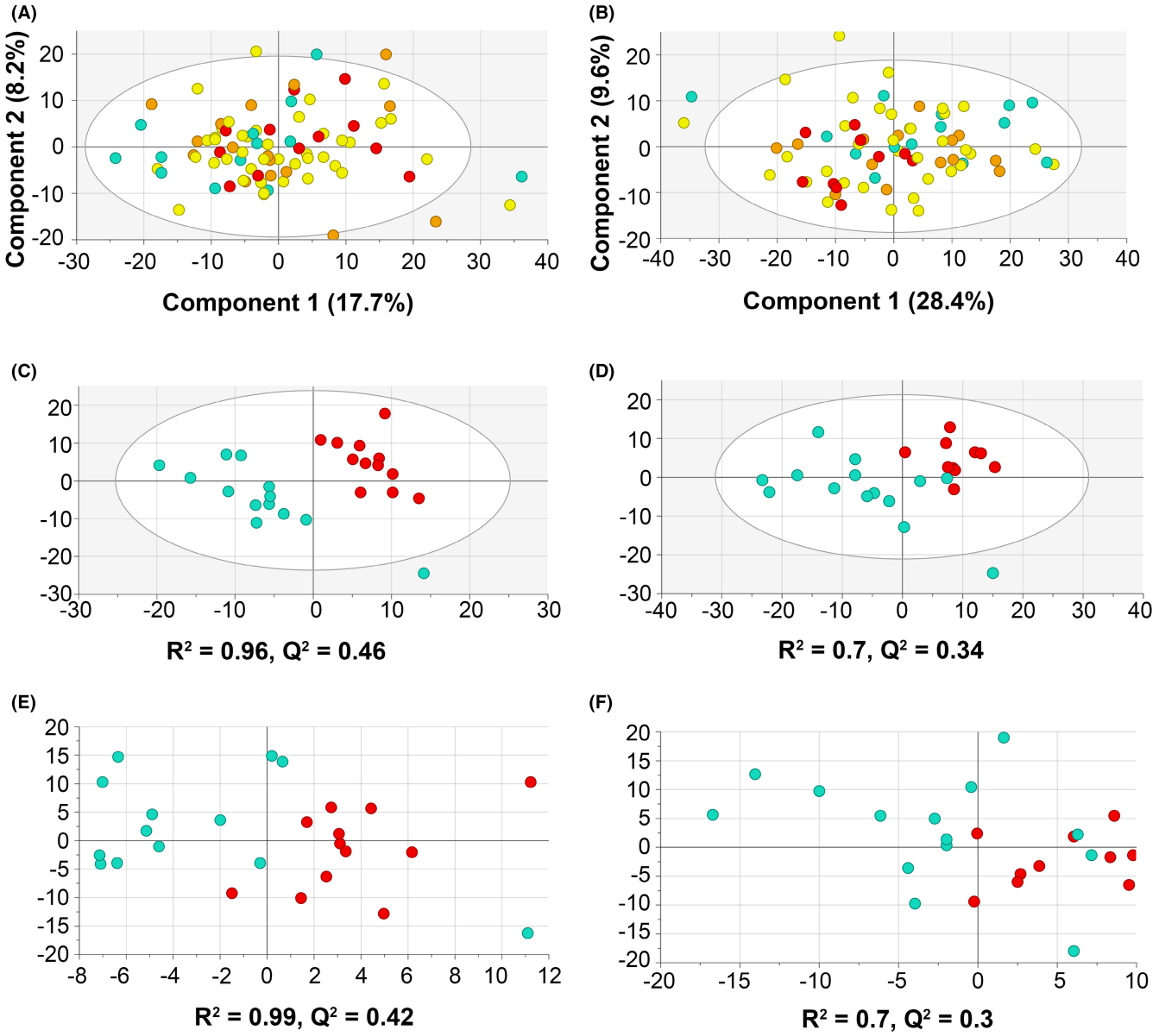


FIGURE 1 Serum metabolomic profile of all patients. A. Unsupervised PCA model of all groups (ICS, IT, Bio, UC) built using the 833 signals for ESI+ that compiled with quality criteria. B. Unsupervised PCA model of all groups built using the 565 signals for ESI− that compiled with quality criteria. C. PLS-DA model for patients controlled with non-systemic corticosteroids (ICS) vs patients with uncontrolled asthma (UC) comparison in LC-MS (ESI+). D. PLS-DA model for ICS vs UC comparison in LC-MS (ESI−). E. OPLS-DA model for ICS vs UC comparison in LC-MS (ESI+). F. OPLS-DA model for ICS vs UC comparison in LC-MS (ESI−). All data were UV scaled. (Key: 'ICS': blue, 'IT': yellow, 'BIO': orange, 'UC': red). R^2 is the capability of the model to classify the samples; Q^2 is the capability of the model to predict the class of a new sample

patients were prescribed with anticholinergics (AC); 41.7%, with AH; and 16.7% were having oral corticosteroid (Oral CS), while 8.3% were having theophylline (T). 33.3% were taking topic CS.

3.2 | Severe uncontrolled asthmatic patients display a unique metabolic fingerprint

The metabolomic signature of each patient was obtained by LC-MS. A total of 833 signals for ESI+ and 565 for ESI− complied with the quality criteria for metabolomics. Clustering of the QC injections in the non-supervised (PCA) model indicated the high quality of the data, while dispersion of the samples showed the biological variability of the patients (Figure S1).

Patterns of the groups were analysed by PCA models for ESI+ and ESI− modes, respectively (Figure 1A,B). UC patients clustered together, especially in the ESI− mode. Additionally, we performed PCA models to see the differences between the UC group and the others, but only differences between ICS and UC patients were observed (Figure S2).

More restrictive analysis was performed to assess the metabolic differences between ICS and UC patients, the ones that differed most

in terms of asthma control. To evaluate real differences between ICS and UC groups, PLS-DA and OPLS-DA models were performed (Figure 1C–F). The robustness of OPLS-DA models was assessed by a seven-fold cross-validation.[16] The resulting models showed good quality parameters: R^2 (0.70–0.96) for the variance explained, and Q^2 (0.3–0.46) for the prediction capability by the model. The cross-validated OPLS-DA scores plot showed that the percentage of accuracy was of 84% and 75% for ESI+ and ESI− modes (Figure 1D–E), respectively. From these models two ICS patients were observed to be outliers (bottom right in the panels from Figure 1C,F). The outlier patient from the ESI+ mode has the lowest FEV1 and FVC of the group (≈70%), while the outlier patient from the ESI− mode has hypersensitivity to NSAIDs drugs. However, we did not find any remarkable reason to exclude these patients from the study.

Furthermore, as age was found significant between ICS and UC groups, PCA models were performed to illustrate their significance (Figure S3). We found that only for the ESI− mode there is a slightly trend of clustering inside ICS group between younger or older than 35 years old.

Together, these results reveal that the UC group is different from the ICS group in terms of their metabolic fingerprint. However, potential differences with IT and BIO might be expected.

3.3 | Severe uncontrolled asthma metabolic fingerprint is characterized by increased levels of lipid mediators

Univariate analysis was carried out to obtain significant metabolic signals in the ICS vs UC comparison, finding 280 with $p < 0.05$ (204 of them had a FDR < 0.2). Using MS/MS fragmentation experiments, we could annotate (identify) 89 of these metabolites. Most of them had a FDR < 0.2 (83 out of 89); which is valid in exploratory studies as it allows to find more potential biomarkers.[17-19]

Next, aiming to see if the metabolic signals were able to stratify the patients according to their group, we built a heatmap with hierarchical clustering analysis (HCA) (Figure 2) using the 280 statistically significant signals ($p < 0.05$). We found that ICS patients (in light blue) are stratified at the bottom of the heatmap, while UC patients (in red) are located in the top. Accordingly, ICS and UC patients are able to be clustered separately using these 280 signals, as expected. IT and BIO patients did not cluster in any specific part of the heatmap, although most BIO patients seemed to be closer to UC patients. Therefore, we demonstrate significant metabolic features that differentiate ICS and UC and point out specific features for IT and BIO patients, although no significant clustering was demonstrated.

Since age was significant between ICS and UC groups (Table 1 and Figure S2), ANCOVA was used to remove those metabolites that were affected by age. Therefore, we ended up with 86 annotated metabolites that were not affected by age. ANCOVA test, physicochemical and statistical details of annotated metabolites are shown in Tables S2-S4, respectively. From these, we obtained 33 metabolites that were uniquely identified.

Among the significant annotated metabolites there were amino acids, bile acids, fatty acids, phospholipids, sphingolipids and vitamins; with phospholipids being the greatest group. Changes in the abundance of compounds between ICS and UC are presented in Figure 3 and Figure S4.

Most of these compounds, such as lysophosphocholines (LPC), lysophosphoethanolamines (LPE), lysophosphatidylinositols (LPI), leucine, arginine, arachidonic acid, sphingosine-1-phosphate (S1P) and retinol were increased in the UC group; with the exception of bilirubin, deoxycholic acid (Figure S4) and phosphocholine 16:0/20:5 (Figure 3), which were decreased in the UC group.

We then performed a pathway analysis of the unique annotated metabolites ($n = 33$) to look for the routes in which they are involved (Table S5). We found an altered phospholipase A2 (PLA2) pathway (100% of affected metabolites in this route, whose changes are shown in Figure 4), along with arachidonic acid metabolism (79.81%), lipoprotein remodelling (52%), phospholipid biosynthesis (95.83%), sphingolipid metabolism (100%) and biosynthesis (82.86%), and other lipid-related routes (Table S5). There are also changes in signalling and transport pathways. Interestingly, there is an increase in metabolites related to a deficiency in leukotriene C4 production (91.5%). These routes are significantly enriched in the UC compared to the ICS patients.

Figure 5 represents metabolites from oleic acid and arachidonic acid pathways. We observed that all measured metabolites that participate in these pathways are increased in UC patients. Thus, we conclude that UC patients have an altered lipid metabolism, with increased levels of lipid mediators in serum compared to the ICS group.

3.4 | UC patients have a distinctive expression of inflammatory-related proteins

In addition to the metabolic fingerprint, we studied the protein serum profile in ICS and UC patients. We measured the expression of 92 proteins (Table S6) and found that 8 of them were significantly different between ICS and UC ($p < 0.05$) (Figure 6A). From these, one of them (CASP-8) also had FDR < 0.2 (Table S6). A STRING analysis was performed in order to find relations between the differentially expressed proteins. We were able to link 7 out of the 8 proteins (Figure 6B). We found that CCL13, ARG1, IL15, TNFRSF12A and CASP-8 were increased in the UC patients, whereas sCD4, CCL19 and IFNγ were decreased (Figure 6C).

Finally, we looked for biological processes in which these proteins were involved using gene ontology (GO) pathway enrichment analysis (Table S7). We found that these proteins are linked to immune response including cytokine response, leukocyte differentiation and adhesion, T cell activation, and nitrogen metabolism, among other pathways.

Therefore, proteomic results support that UC patients have an altered inflammatory response, and point out immune cell activation as a possible key mechanism.

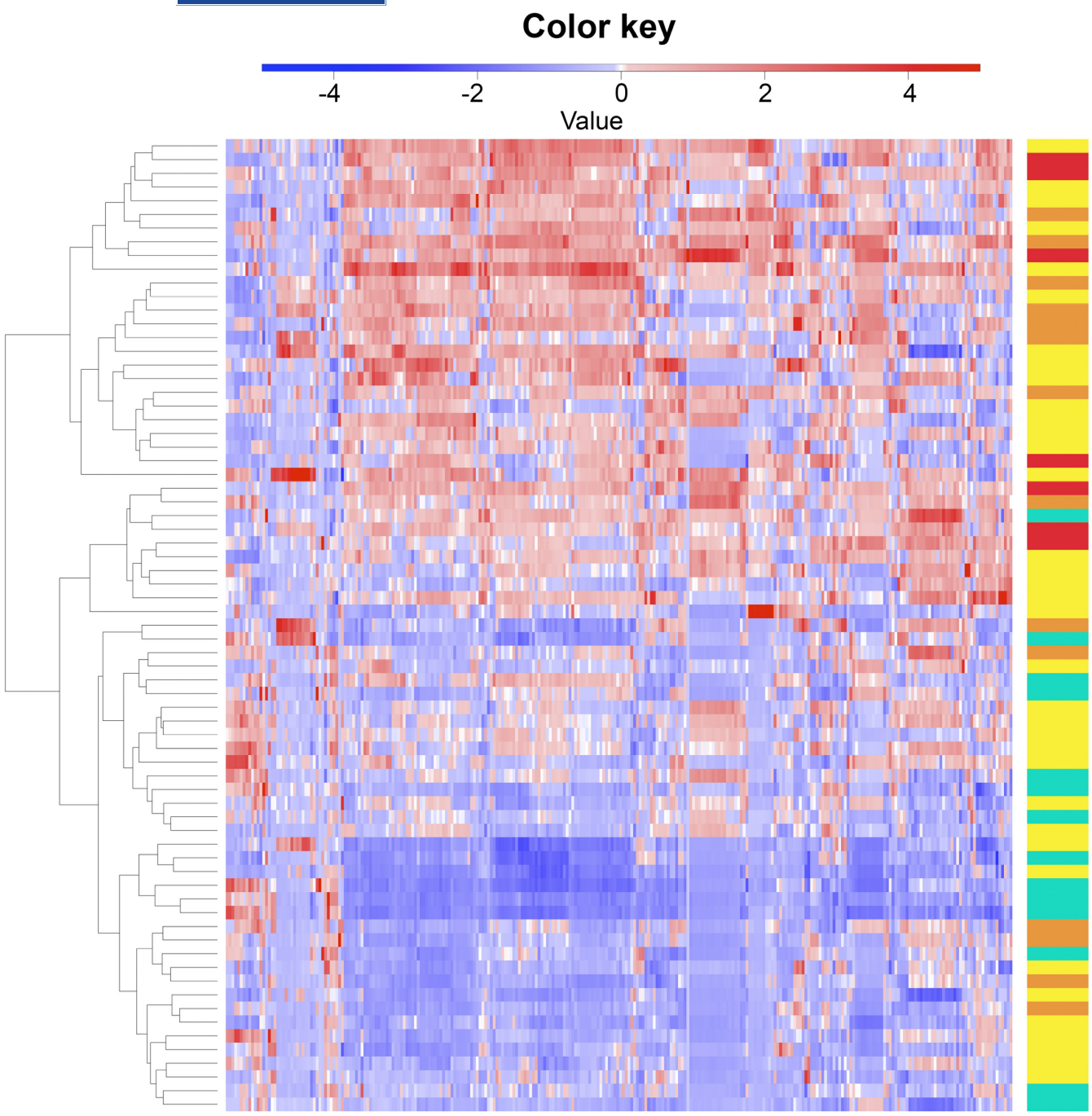


FIGURE 2 Hierarchical clustering heatmap of the patients from the four groups (in rows) and the 280 significant metabolites between ICS and UC (in columns). Red cells represent an increase of the specific metabolite in that sample, whereas blue cells represent decreased metabolites. Samples and metabolites are clustered according to their similarity, and the group they belong to is showed with colours at the right. (Key: 'ICS': light blue; 'IT': yellow; 'BIO': orange; 'UC': red)

3.5 | Machine-learning models for UC classification

Aiming to obtain a more accurate profile of the UC asthmatic phenotype, we integrated both the metabolomic and clinical data in a ML model. Since the proteomic analysis was performed in a limited number of samples, it could not be included in the model. The ML model aim was to find which variables could be used to classify UC and ICS patients. The k-NN model is a ML algorithm that classify a sample based on all the variables given, which has been tested for breast cancer prediction model.[20] The k-NN learning algorithm requires us to specify how many neighbours (*k*) are going to be considered. To obtain this parameter we used the elbow method. The lack of elbow when using only clinical or metabolomic data alone suggests that these datasets are not good enough to predict classification of UC and ICS patients on their own. When using both datasets combined, the elbow test suggested that the *k* parameter should be either 2 or

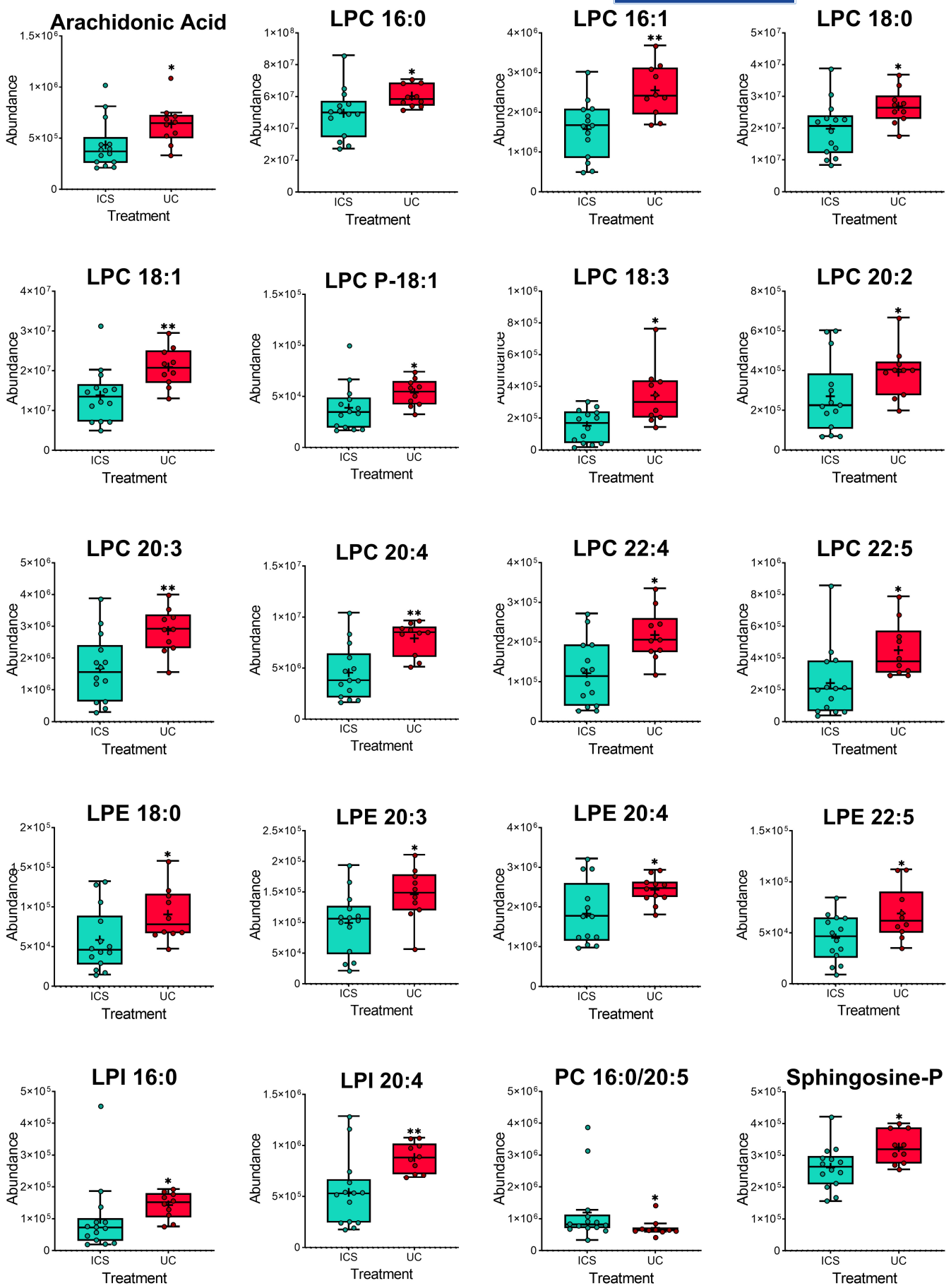


FIGURE 3 Legend on next page

FIGURE 3 Trajectories of the most relevant identified compounds. Box and whiskers plots with mean are represented for corticosteroid-controlled (ICS, blue) and uncontrolled (UC, red) groups. For metabolites that had more than one *m/z* or that were detected in both ESI modes, the most abundant *m/z* overall was represented. Mean is represented with '+' inside the boxes and individual data points are shown as blue (ICS) or red (UC) dots. Mann-Witney *U* test was used to calculate significant differences. $^{*}p < .05$; $^{**}p < .01$. Other trajectories can be found in Figure S4

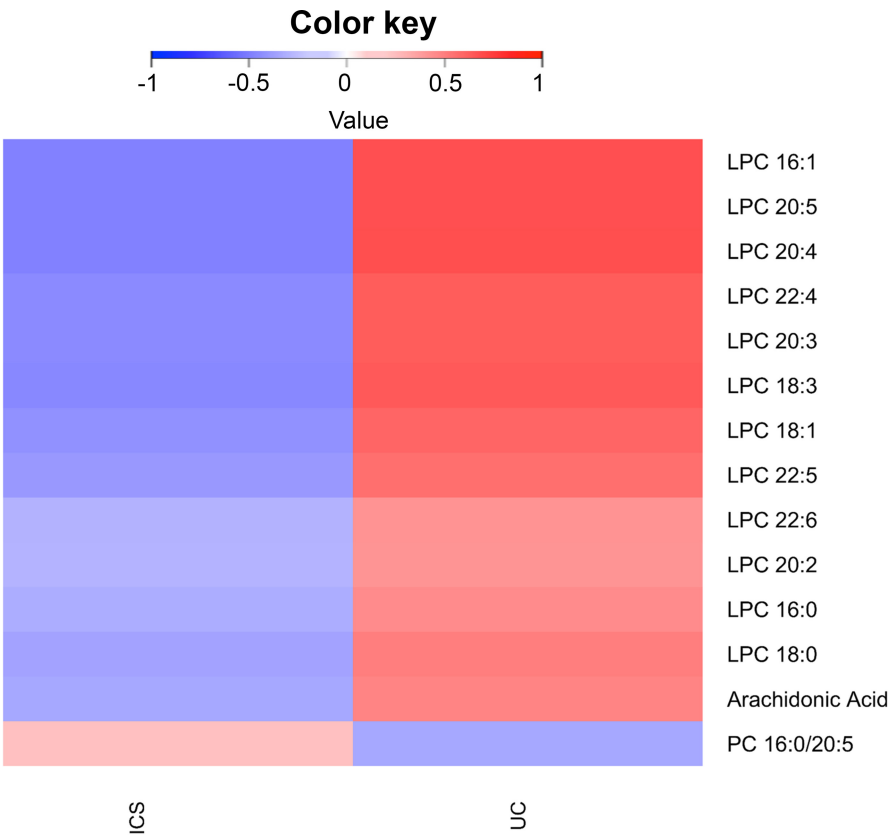


FIGURE 4 Changes in the abundance of metabolites in the PLA2 pathway between ICS and UC patients. Red cells represent an increase in the amount of a metabolite, whereas blue cells represent a decrease. *LPC*: *lysophosphocholine*, *PC*: *phosphocholine*

3, so we built one model with each value to select the one with a better performance. However, both models ended up being identical.

From the original 136 variables (50 clinical variables and 86 metabolites), we ended with a k-NN model that used 39 (16 clinical and 23 metabolomic variables). This model was able to correctly classify all patients but one. The accuracy, specificity, sensibility, positive predicted value (PPV) and negative predicted value (NPV) were all ≥ 0.75 (Table S8). We also performed a variable selection analysis, but the resultant model had worst performance (data not shown).

4 | DISCUSSION

Management of uncontrolled severe asthma entails a significant challenge for healthcare professionals. These patients are difficult to identify in the first place and need personalized treatment because they do not respond to conventional pharmacological interventions. We believe that a better knowledge regarding the metabolic, proteomic and biological pathways associated with this specific phenotype will provide new insights and will be helpful towards the management of these patients.

Here, we studied a cohort of HDM-allergic asthmatic patients from a high-exposure HDM region. The Canary Islands are located near the west coast of North Africa, close to the Sahara Desert, and thus, are impacted by Saharan Dust (calima) during approximately 30% of the year. The most common HDM sensitizers in the Canary Islands are *Dermatophagoides pteronyssinus* and *Blomia tropicalis*, which are linked to asthma prevalence.[7,21] Moreover, prevalence of persistent rhinitis and asthma is higher in this area than in the mainland of Spain, as previously reported,[6,11] probably due to the higher sensitization profile to perennial allergens and favourable ecological conditions supporting mite growth. Similar conditions have been described in other insular areas like the United Kingdom, Australia or New Zealand.[7,12] Thus, even if this study was carried out in a specific region, the results can be extrapolated to many Atlantic climate areas.

Moreover, this cohort of patients is characterized by a well-known exposome and a recruitment process by a single clinical group with more than 30 years of experience in the field, which assures the uniformity of inclusion criteria and treatment strategy. This fact minimizes variability factors, making this a unique asthma model that is representative of the severe allergic asthma phenotype.

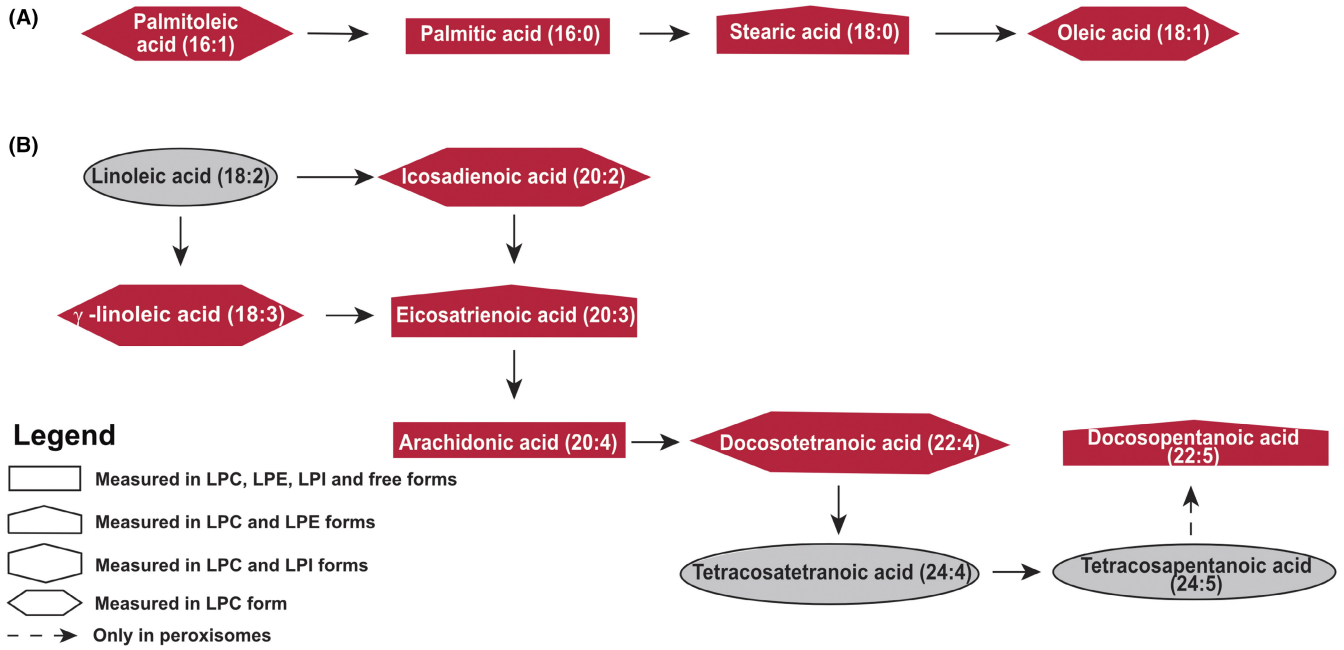


FIGURE 5 Pathways of lipid metabolism in uncontrolled patients. A. Oleic acid pathway; B. Arachidonic acid pathway. In red, metabolites increased in uncontrolled patients (UC) with respect to corticosteroid-controlled patients (ICS); in grey, metabolites that were not significantly changed between UC and ICS patients

According to their response to treatment, patients were classified into 4 groups. Patients that controlled asthma with non-systemic CS were considered mild HDM-allergic asthmatic patients (ICS); and patients that do not respond to none of the available treatments are considered uncontrolled HDM-allergic asthmatic patients (UC). Additionally, patients controlled with IT and biological drugs were also included, showing a disperse distribution probably due to several factors, like time of treatment, administration pathway or response to treatment, that might need further analysis. Moreover, BIO patients were only treated with omalizumab, since it was the only biological drug approved for the treatment of asthma in the Canary Islands at the time. Therefore, it will be relevant to perform further studies considering other biologics such as mepolizumab or benralizumab.

Here, we combine an appropriate clinical classification, focusing on disease control, with a wide range of techniques to identify metabolites and proteins.

Metabolomics is a promising tool in the detection of dynamic changes and alterations in the metabolism associated to a pathology.[22] In fact, the metabolome has been described to be closely linked to the phenotype of a disease and can be an extremely useful tool when evaluating the effect of treatments. From a practical point of view, it uses very sensitive and specific techniques, such as LC-MS, which allows the simultaneous detection of a great variety of metabolites in each biological sample.[23,24]

In this study, we performed an untargeted metabolomic analysis in all samples from each experimental group, what results, after a complex and laborious statistical analysis, into the identification of several significant signals that conform the metabolomic signature of UC group. Therefore, this study should be understood as exploratory. In addition to the metabolites, we have also identified the biological pathways associated to those metabolic changes. However, further analyses in prospective studies are needed to validate these metabolites as potential biomarkers in a new and larger cohort of patients, including other subtypes of asthma.

Most of the identified compounds (94%) have a FDR < 0.2. For exploratory studies, setting higher cut-offs allows them to find more potential biomarkers resulting in a more extensive knowledge[17-19]; although validation in further studies is critical to ensure that the resulting biomarkers can properly classify the patients.[25]

Our metabolomic results show that LPC, LPE and LPI, along with arachidonic acid, are increased in the UC compared to the ICS. Lipid metabolism has been previously associated with asthma, as lipid mediators are related to inflammatory signalling pathways. Yoder *et al*[26] previously reported increased levels of LPC 16:0 and LPC 18:0 in the bronchoalveolar lavage fluid (BALF) of patients with moderate asthma compared to non-asthmatic or mild-asthmatic patients. This was also accompanied by an increase in PLA2 activity, a pathway that we report increased in UC patients as well. Moreover, Pang *et al*[27] compared eosinophilic and non-eosinophilic asthma, finding that LPC 18:1 was increased in the eosinophilic phenotype. As a matter of fact, LPC have been reported to have a role in eosinophil regulation.[28-30] Therefore, the observed increase in LPC seems to be directly associated with an exacerbated inflammatory response taking place in UC patients.

LPC have also been associated to arachidonic acid release in human derived monocytes.[31] Arachidonic acid (C 20:4; AA), eicosapentaenoic acid (C 20:5; EPA) and docosahexaenoic acid (C 22:6; DHA) were detected either as free fatty acids or in LPC, LPE and/or LPI forms. An enrichment of these metabolites and their implied

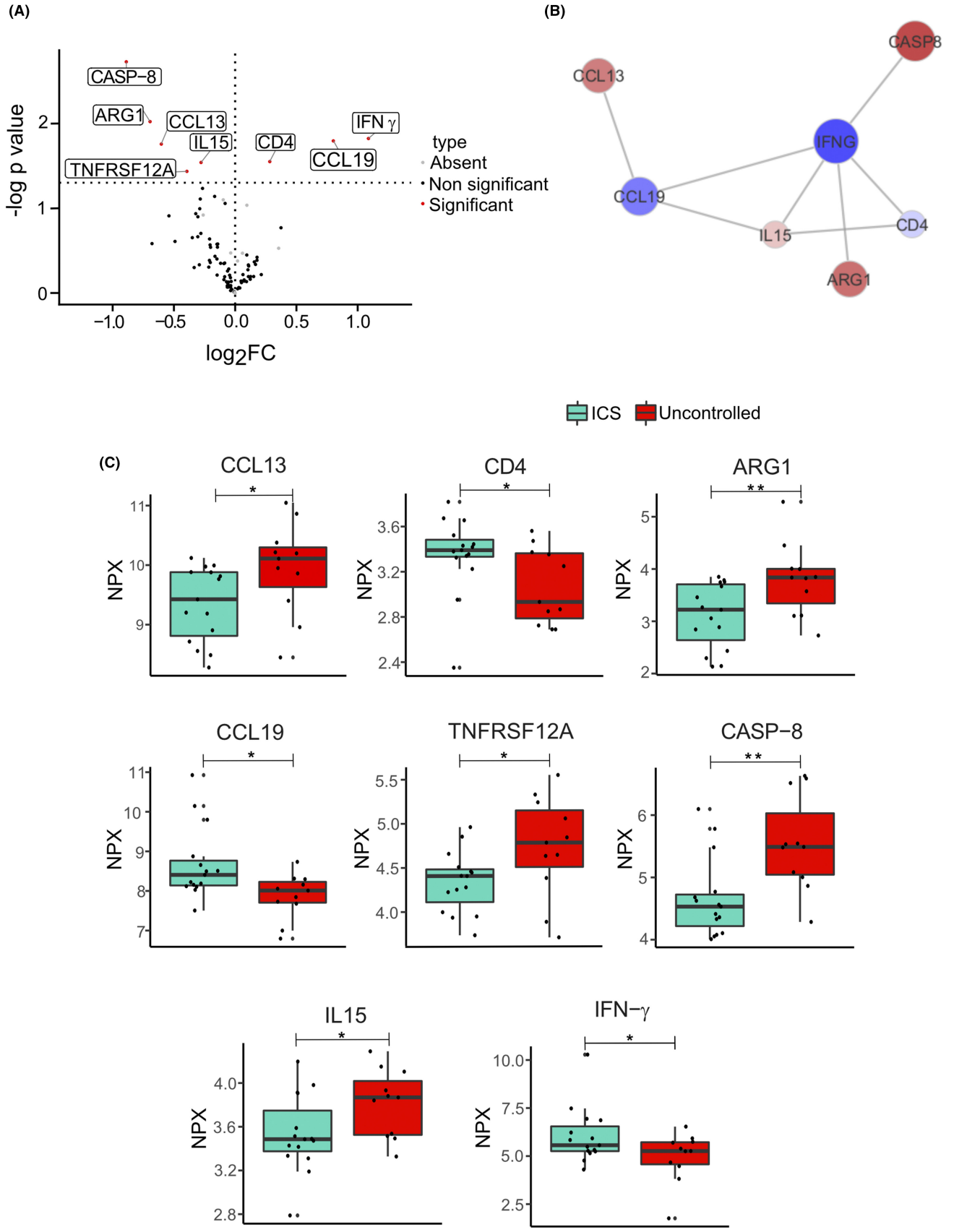


FIGURE 6 Specific serum proteomic profile of ICS and UC patients. A. Volcano plot of the differences in proteomic profile between ICS and uncontrolled patients. B. STRING analysis of significant proteins. An increase in the size of the circles and intensity of colour signals an increase in logFC. Red signals an increase of the protein expression, while blue means that the protein is decreased. C. Boxplot of the relative abundances of significant proteins between ICS (light blue) and UC (red). Significance $^{*}p < .05$, $^{**}p < .01$

pathways has been previously reported in asthma.[27,32-34] These fatty acids act as precursors of eicosanoids (prostaglandins, leukotrienes, lipoxins), resolvins and protectins through the activity of enzymes such as cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450.[35,36] This suggests that UC patients have a significant activation of these pathways, which are involved in the regulation of the immune response.

S1P was also increased in UC patients. This metabolite has been previously associated with asthma and other lung inflammatory diseases,[37] along with asthma severity.[32,38] Interestingly, we have reported an increase of S1P levels in plasma of patients with severe allergy.[39] Moreover, S1P also plays a key role in regulation of T cells lifespan and recruitment.[40] Thus, this increase might point to the activation of T cells in UC patients, which may play a role in uncontrolled asthma pathogenesis.

Other metabolites significantly altered in UC patients are arginine, which is related to the nitric oxide (NO) pathway[38,41-44]; and leucine, associated with the activation of mTORC1 pathways responsible for T cells activation, proliferation and differentiation.[45]

Furthermore, proteomic analysis reveal that UC patients seems to have an increased pro-inflammatory T cell response, which is also supported but the changes in S1P and leucine abovementioned. We found an increase in CCL13 and IL-15 in the UC patients, which have been shown to attract monocytes and lymphocytes and promote T cell proliferation, suggesting an activation of the immune response.[46-49] This activation would be mainly produced in the mucosa, as there was a reduction of CCL19, responsible of lymphoid organ homing, and soluble CD4.[50-52] Moreover, this is a type 2 response, as shown by the decrease of IFNγ. This inflammation was accompanied by complementary epithelial and endothelial permeability and growth, as shown in the increase of proangiogenic factor TNFRSF12A and ARG 1, which regulates NOS activity.[53,54] Finally, this inflammation might be maintained in time by the increased levels of CASP-8, as this caspase is important to maintain T cell proliferation and has been shown to inactivate RIPK1, which limits TNF and inflammatory responses.[55,56]

Together, metabolomic and proteomic data demonstrate unique biological mechanisms associated with HDM-allergic asthmatic uncontrolled asthma patients (UC) when compare with the mild phenotype (ICS), which are mainly associated with a pro-inflammatory environment and T cell activation and proliferation.

Next, aiming to integrate clinical and demographical with metabolic data and to identify potential classifiers for UC patients, we performed a ML approach. K-NN algorithm is one of the simplest ML algorithms, but it has a very efficient performance.[57] Using this model, we could properly classify UC patients with more than 80% of accuracy. In fact, our results demonstrate that inclusion of clinical data in the ML model significantly improved patient's classification, demonstrating the need to develop more bioinformatic models that allow the integration of clinical and omics data to generate a complete picture of the patient phenotype.

Currently, high throughput techniques are already being used in other disciplines, like cancer or even in allergy (*e.g.* protein arrays). The next step in precision medicine will be to have a more detailed and accurate vision of the patient through the integration of data from one or more high throughput techniques (multi-omics) with clinical and demographical data. This type of analysis will provide a better comprehension of the pathology, and will help develop better systems to classify patients, design personalized treatments and reach a more accurate diagnosis.

Together, these data show a set of specific biological features associated with the UC phenotype. Metabolomics, proteomics and clinical data all point towards a systemic and sustained inflammatory response, underlined by an exacerbated lipid, S1P and NO metabolism, and an increase in T cells activation and proliferation. Moreover, our results support the need of exhaustive clinical criteria to better characterize and classify these patients. These observations shed light into the molecular and cellular mechanisms underlying this phenotype and provide a starting point to design novel personalized treatments for these patients.

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CONFLICT OF INTEREST

Delgado-Dolset has nothing to disclose. Dr. Obeso has nothing to disclose. Rodriguez-Coira has nothing to disclose. Dr. Tarín has nothing to disclose. Dr. Tan has nothing to disclose. Dr. Cumplido Bonny has nothing to disclose. Dr. Cabrera Santana has nothing to disclose. Dr. Angulo Díaz-Parreño has nothing to disclose. Dr. Barbas reports grants from Ministerio de Ciencia, Innovación y Universidades co-financed with FEDER RTI2018-095166-B-I00, during the conduct of the study. Dr. Sokolowska reports grants from Swiss National Science Foundation (SNSF), grants from GSK, outside the submitted work. Dr. Barber reports grants from ALK, Allero Therapeutics, personal fees from ALK, AIMMUNE, outside the submitted work and

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AUTHOR CONTRIBUTIONS

MME was the PI and together with DB, AV, CB and MS designed and supervised the research. TC, JAC and AC recruited the patients and obtained the samples. MIDD, JR-C, AV and DO performed the metabolomics analysis and data treatment. JR-C and GT performed the proteomic analysis and data treatment. MIDD performed analysis of the results together with CT and SA. All authors contributed to the writing of the manuscript and have given approval to the final version of the manuscript.

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