**U-BIOPRED clinical adult asthma clusters linked to a subset of sputum *-omics***

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## **ABSTRACT 246 w**

### **Background:** Asthma is a heterogeneous disease in which there is a differential response to asthma treatments. This heterogeneity needs to be evaluated so that a personalised management approach can be provided.

### **Objectives:** We stratified patients with moderate-to-severe asthma based on clinico-physiological parameters and performed an *-omics* analysis of sputum.

### **Methods:** Partition-around-medoid clustering was applied to a training set of 266 asthma participants from the European U-BIOPRED adult cohort using 8 pre-specified clinic-physiological variables. This was repeated in a separate validation set of 152 asthmatics. The clusters were compared based on sputum proteomic and transcriptomic data.

### **Results:** Four reproducible and stable clusters of asthmatics were identified. The training set cluster T1 consists of well-controlled moderate-to-severe asthmatics, while cluster T2 is a group of late-onset severe asthmatics with history of smoking and chronic airflow obstruction. Cluster T3 is similar to cluster T2 in terms of chronic airflow obstruction but is composed of non-smokers. Cluster T4 is predominantly composed of obese female uncontrolled severe asthmatics with increased exacerbations, but with normal lung function. The validation set exhibited similar clusters, demonstrating reproducibility of the classification. There were significant differences in sputum proteomics and transcriptomics between the clusters. The severe asthma clusters, T2, T3 and T4, had higher sputum eosinophilia than T1 with no differences in sputum neutrophil counts, exhaled nitric oxide and serum IgE levels.

### **Conclusion:** Clustering based on clinico-physiological parameters yielded 4 stable and reproducible clusters that associate with different pathobiological pathways.

1. **Clinical Implications:** The definition of four distinct clusters of asthma linked to different pathobiological pathways provides a better template for the phenotyping and personalised treatment of severe asthma, where high unmet needs remain.
2. **Capsule Summary:** Unsupervised clustering of asthma on clinical features alone has led to the definition of four phenotypes. Sputum ‘omics’ analysis has revealed different biological pathways pointing towards potential new treatments.
3. **Key words:** Severe asthma, clustering, sputum eosinophilia, partition-around-medoids algorithm
4. **Abbreviations:**

**ACQ:** Asthma Control Questionnaire

**ANOVA:** ANalysis Of VAriance

**BMI:** Body Mass Index

**CDF**: Cumulative Distribution Function

**COPD:** Chronic Obstructive Pulmonary Disease

**FDR:** False Discovery Rate

**FeNO:** Fractional exhaled Nitric Oxide

**FEV1:** Forced Expiratory Volume in 1 second

**FVC:** Force Vital Capacity

**ICS:** Inhaled CorticoSteroids

**IgE:** Immunoglobulin E

**OCS:** Oral CorticoSteroids

**PAM:** Partition-Around-Medoids

**SARP:** Severe Asthma Research Program

**Th2:** Lymphocyte T-helper 2

**U-BIOPRED:** Unbiased BIOmarkers for the PREDiction of respiratory diseases outcomes**INTRODUCTION**

Although clinicians have been focusing on the definition and classification of asthma severity and disease risk for the past decade, there is now a consensus that a deeper understanding of the basis of the heterogeneity of asthma is necessary in order to find targeted treatments for specific asthma phenotypes1. This is imperative for patients with severe asthma because this group of patients does not fully respond to currently-available asthma medications1 and is likely to constitute of a number of different asthma phenotypes2. There is therefore a need to improve the identification and definition of these phenotypes of asthma.

Cluster analysis using unsupervised statistical approaches has already led to the definition of clusters on the basis of similarities in clinical and inflammatory biomarkers3, 4. However, these studies have used relatively homogeneous populations and so may not reflect the real-life situation. One example is the exclusion of current or previous smokers with asthma, a group that may have an asthma-COPD overlap syndrome5. In addition, previously-derived clusters have not been linked to underlying biological profiles apart from the use of blood or sputum eosinophil counts. Other approaches have been to use unsupervised gene and protein omics data to cluster patients with asthma 6-8.

In this study, we used a robust clustering approach using clinical and physiological parameters that are available to the asthma physician in a broad range of participants with mild/moderate to severe asthma, including smokers and ex-smokers recruited in the U-BIOPRED project9. The second step was to explore the underlying pathobiological pathways of these clusters by examining the differential expression of the transcriptome of sputum cells and the proteome of sputum supernatants that exist between the clusters generated to determine whether they exhibit any differences in specific pathobiological pathways.

**METHODS**

### **U-BIOPRED cohorts**

We used a subset of the U-BIOPRED adult baseline data. The U-BIOPRED cohort comprises 509 asthmatics, both mild-moderate and severe and including non-smokers, ex-smokers and current smokers and 101 non-asthmatic control subjects. They had undergone detailed phenotypic characterisation using established standard operating procedures, as described previously9. The study participants were split randomly into training and validation datasets in a 2:1 ratio with the two groups being balanced in terms of asthma severity, age and gender. The validation group was used for internal replication. All participants gave signed informed consent to participate in the study which was approved by National Ethics Committees.

#### Clinical variables

The cluster analysis was focused on key variables that are readily accessible to the general practitioner representing important historical, clinical and physiological parameters underlying each participant with asthma. These variables were: age of onset of asthma symptoms, pack-years of cigarette smoking, body mass index (BMI), forced exhaled volume in 1 second (FEV1) as percentage of predicted value (FEV1% predicted), FEV1/FVC ratio (FVC: forced vital capacity), the average score of the 5 first questions of the Asthma Control Questionnaire (ACQ-5), self-reported numbers of exacerbations in the previous year and the daily dose of oral prednisolone or equivalent.

### **Data pre-processing and cluster analysis**

Box-Cox power transformation10 was used to approximate the data to a normal distribution using the powerTransform function from the R package *car* 11, which uses maximum likelihood to determine the best lambda. Data was then centred-scaled in order to ensure similar ranges for all the parameters and reduced using principal component analysis (PCA) ensuring that there was no correlation between the composite variables, thereby avoiding skewing of the analysis.

Clustering schemes are descriptive methods that group participants with similar characteristics. To determine similarity between participants, the Euclidean distance (which actually measures dissimilarity using the ordinary straight-line distance between 2 points) was used. Clustering was performed using the partition-around-medoid (PAM) algorithm, a more robust generalisation of the k-means method12. To assess the stability of clusters, bootstrapping (also known as consensus clustering) was performed by randomly removing 10% of the data and repeating the clustering for a total of 1,000 times13. The stability of the clusters was assessed by studying the cumulative distribution function (CDF) which, as represented in Figure 1A, describes the proportion of pairs of participants (on the y axis) that are clustered together in at most x percent of bootstrap iterations (on the x axis). Thus, if the curve is flat for example between x values of 0.2 and 0.8, this means that there are no pairs of participants that are clustered together between 20 and 80% of the iterations; *i.e.* they are almost never clustered together (<20%) or almost always clustered together (>80%). Clustering results were considered stable when the middle part of the CDF was flat.

To further define the stability of the clusters, we used an in-house objective called ‘deviation from ideal stability’ (Figure E1 in the Online Repository), which is at its best when it is close to zero. Additionally, to gain confidence in the existence of these clusters14, internal validity was checked using the Calinski and Harabasz index15, which measures the ratio of the between-clusters variance to the within-cluster variance such that the higher the value, the better defined are the clusters.

### **Sputum induction, transcriptomics and protein analytes**

### Sputum induction was performed following inhalation of hypertonic (0.9 to 4.5%) saline using a DeVilbiss 2000 Ultrasonic nebuliser (Somerset, PA, USA) according to a standardised protocol16. Sputum plugs were selected and liquefied using dithioerythritol. Differential cell counts were determined by assessment of a maximum of 500 to 1,000 inflammatory cells on Diff-Quick stained cytospins. Cytospin assessments were performed centrally with the outcome of the cytospin analysis determining the suitability of the sample for analysis by accepting only those samples with a cell viability of ≥ 50% and squamous cells of ≤ 40%.

Transcriptomic analysis was performed using the Affymetrix® HT HG-U133+ PM GeneChip on extracted RNA from sputum cells derived from cell pellets with a specific cut-off of ≤ 30% squamous cells. Technical and biological quality checks were performed following Affymetrix® recommendations with only RNA samples of high purity (RIN > 6.5) used for amplification; raw data were pre-processed using the robust microarray analysis (RMA) method from the *affy* R package17 to derive the expression matrix.

### From each one of the frozen aliquots of sputum supernatant, 1,129 analytes were quantified using the SomaScan™ v3 platform (SomaLogic®, Boulder, CO; *www.somalogic.com*) using SOMAmer® (Slow Off-rate Modified Aptamer) protein-binding reagents. These assays combine the best properties of antibodies and traditional aptamers, which are highly specific for the corresponding cognate proteins18. Analyte levels were reported as relative fluorescence units, cross-plate calibrated, and median normalised.

### **Statistical analysis**

All analyses were undertaken using the R software for statistical computing (version 3.1.2). Clinical variables were compared between clusters using analysis of variance (ANOVA) for multiple group comparison of normally-distributed variables. The Kruskal-Wallis test was used for multiple group comparison of ordered categorical or non-normally distributed variables and the χ² test was used for qualitative variables. To compare protein abundance or transcript expression, an ANOVA test was performed on the data (transformed with base 2 logarithm) adjusting for age and gender followed by a Tukey post-hoc pairwise comparison test. Protein analytes or probesets were defined to be consistently differentially abundant or expressed when their respective p-values were below 0.05 in both the training and validation sets analysed separately and when these sets were analysed together (preventing the inclusion of features that would have a different direction of change in the training and the validation sets). This allows for a reproducible and relatively stringent features selection process, and lowering the false positive rate despite not correcting the p-value for multiple testing. Both proteomics and transcriptomics datasets have been checked for any batch or site effect and corrected accordingly using ComBat method19.

Pathway enrichment analysis was performed using the results of the statistical analysis described above. The lists of contrast-specific features consistently found in both training and validation sets were submitted to the *g:Profiler* web-tool for enrichment analysis20. The p-values for the enrichment analysis were corrected for false discovery rate (FDR) with the Benjamini-Hochberg method21. Feature lists for each comparison were tested for enrichment against the KEGG22 and Reactome23 databases.

**RESULTS**

### **Participants**

### A total of 418 asthmatics out of 509 with a complete set of data for the 8 variables were available for analysis and were split into training (n=266) and validation (n=152) sets. The distribution of asthma severity, age and gender, and all 8 variables included in the clustering for the training and validation sets were not statistically different between the two sets although FEV1 (% predicted) and daily dose of oral corticosteroids (OCS) were incompletely balanced (p-values of 0.07 and 0.06 respectively) (Online Repository Table E1).

### **Training set clusters**

Consensus clustering on the training set was run in order to assess stability for a number of potential cluster numbers varying from two to ten. This resulted in the separation of two or four stable groups after resampling as defined by a flat middle part of the consensus CDF13 (Figure 1A), well-defined squares within the consensus matrix (Figure 1B) and by minimal values for the deviation from ideal stability index (Figure 1C top). These cluster numbers were also associated with the two highest Calinski and Harabasz indices (Figure 1C bottom), indicating that the clusters were more compact than the overall data. While separating into two and four clusters resulted in almost similar quality, four clusters were chosen for further analysis (denoted T1 to T4) to allow for a more precise sub-phenotype definition. Indeed, the two cluster allocation mainly regroups T1 with T4 and T2 with T3. Finally Figure 1D represents a heat-map of distances between the participants in the four clusters.

**Four-cluster analysis (T1 to T4)**

The four clusters are described in Table 1 and in Online Repository Table E2. Briefly, *Cluster T1* is composed of moderate-to-severe well-controlled asthmatics with normal FEV1, low sputum eosinophilia, almost no OCS use (6%) and a high proportion of atopic participants (84.1%). *Cluster T2* is mainly composed of overweight to obese (79% with BMI ≥ 25 kg/m² and 41% with BMI ≥ 30 kg/m²), late-onset severe asthmatics who smoked, with relatively poor control, severe airflow obstruction (mean FEV1: 58.9% predicted), and had the highest sputum and blood eosinophilia, with a lower proportion of atopic participants than in the other three clusters (55.6%). *Cluster T3* is similar to Cluster T2 except that the asthmatics were non-smokers, were less overweight, had poorer lung function and a higher proportion of atopic participants (70.6%). *Cluster T4* is mostly composed of obese female asthmatics (83% female, 88% with BMI ≥ 25 kg/m² and 56% with BMI ≥ 30 kg/m²), experiencing frequent exacerbations with poor asthma quality of life despite near normal lung function and 73.6% of positive atopy status. Fractional exhaled nitric oxide (FeNO) and serum immunoglobulin E (IgE) were not differentially distributed amongst the 4 clusters.

### **Validation set clusters**

### The same analysis was done on the validation set. It yielded five relatively stable clusters after resampling (denoted V1, V2, V3, V4a and V4b to align with the training set) as shown by a flat CDF and a low deviation from ideal stability (see Online Repository Figure E2 and Table E3). The Calinsky and Harasbaz index was slightly better for 4 clusters. The difference in the number of clusters compared to the training set might be due to the fact that the validation set was smaller. When comparing the training and validation clusters using the least statistical differences of clinical variables, cluster V1 was found to be similar to cluster T1, V2 to T2 and V3 to T3, while V4a combined with V4b was found to be similar to T4 (Online Repository Table E4). For ease of recall, clusters T1 and V1 will be referred to as Phenotype 1, T2 and V2 as Phenotype 2, T3 and V3 as Phenotype 3, T4 and V4a&4b as Phenotype 4.

### The distributions of the main clinical characteristics of the training and validation clusters were similar (Figure 2) with the exception of the V4a and V4b clusters covering 2 different parts of T4. V4a consists of less obese asthmatics associated with later onset of disease, lower OCS use and better asthma control when compared to V4b.

**Algorithm to predict clinical phenotype**

 Support Vector Machine algorithm with a Gaussian radial basis kernel24 was used to predict phenotypes from the 8 clinical parameters. The model was trained on the training set only using a 10 fold cross-validation method to prevent overfitting with *caret25* and *kernlab24* R packages. The prediction model yielded an almost perfect accuracy of 97% on the training set. It predicted phenotype assignment on the validation set and achieved a very good accuracy rate of 86%. An xlsm file has been developed that can be used to predict the clinical phenotype (see Online Repository).

**Biological characterisation in a subset**

The results of proteomic and transcriptomic profiling in sputum samples were compared between the phenotypes to determine if they could represent a useful categorisation of asthma. Due to the fact that not all patients were able to produce any sputum or good-quality sputum for analysis and due to technical quality control, the number of participants used in the proteomic and transcriptomic analyses were 86 and 94, respectively. The clinical profiles of these participants who provided these samples was not different from those of the whole cohort as shown in Online Repository Table E5. Protein data was available in 86 participants (56 in the training set and 30 in the validation set). Ten proteins out of the 1,129 measured were identified as being consistently differentially abundant between phenotypes (Table II). This number of hits was too small to allow for any meaningful pathway enrichment analysis. Sputum transcript expression data was available for 94 participants (56 in the training set and 38 in the validation set). A total of 345 transcripts (291 annotated) were found to be consistently significantly differentially expressed in at least one of the pairwise comparisons between the phenotypes (Online Repository Table E6). Pathway enrichment results are shown in Table III.

#### Differential protein abundance in sputum supernatants

Both the comparison of Phenotype 2 (severe asthma (ex-)smokers) and Phenotype 3 (severe asthma non-smokers) to Phenotype 1 (well-controlled asthma) highlighted IL-16, a natural ligand of CD4 and CD9 that induces preferential migration of human T-regulatory cells26, as being elevated in the more severe phenotypes. Additionally, compared to Phenotype 2, there was greater abundance in Phenotype 1 of (i) CTAP-III (CXCL7), a potent chemoattractant and activator of neutrophils; (ii) GM-CSF, which controls the production, differentiation and function of granulocytes and macrophages; and (iii) Trypsin 2, which degrades the extracellular matrix. On the contrary, HAPLN1, involved in cell adhesion, was less abundant.

Phenotype 3 was associated with reduced levels of Cathepsin G involved in connective tissue remodelling at site of inflammation, as compared to Phenotypes 1 and 4. Moreover, Phenotype 3 also exhibited elevated levels of ARSB, an arylsulfatase involved in cell adhesion and migration regulation, and PSA2, a member of peptidase T1A family, when compared to Phenotype 1. Lastly, LYN kinase and FUT5 (fucosyl transferase 5) were found to be decreased in Phenotype 3 when compared to Phenotype 2.

**Differential transcript expression in sputum cells**

Comparing Phenotype 2 to Phenotype 1 yielded 8 differentially-expressed genes, 2 of which are linked to the haematopoietic cell lineage pathway (CSF1 and CD1B), both being more expressed in Phenotype 2. The comparison of Phenotype 3 to Phenotype 1 highlighted 147 genes, 5 of them encoding the proteins CTSB, PDIA3, CD4, CD74, CALR that are linked to antigen processing and presentation pathway. Pathway enrichment analysis of Phenotype 3 compared to Phenotype 2 revealed pathways related to the regulation of the actin cytoskeleton (ITGB1, ITGB8, FN1, DIAPH2, F2R, ACTN2), and to fibronectin matrix formation (ITGB1, FN1), potentially linked to the effect of smoking in severe asthma. 3 probesets, one of which annotated to a known gene, DAGLB, that encodes for the enzyme diacylglycerol lipase, were highlighted in the comparison of Phenotype 4 to Phenotype 2, therefore no pathway enrichment analysis was done. The comparison of Phenotype 4 to Phenotype 3 revealed 14 differentially-expressed genes including those encoding proteins related to the cell cycle and growth factor regulating pathways (MAPK1, E2F1 and SPRY2) and to the modulation of immune system responses, particularly the interferon signalling pathway (OASL, OAS3 and TRIM14).

## **DISCUSSION**

Using the partition-around-medoid (PAM) clustering algorithm and a bootstrapping method on the large U-BIOPRED cohort of participants with moderate to severe asthma, we have identified 4 clusters of asthma: one composed of well-controlled asthmatics with almost normal lung function but on low to high doses of ICS and the three others of severe asthmatics. Two of the clusters relate to chronic airflow obstruction with one cluster associated with smokers and ex-smokers with late onset asthma who had the highest blood and sputum eosinophil counts, while the third cluster is associated with non-smokers on oral corticosteroid therapy. Finally, the fourth cluster of severe asthma relates to obese female asthmatic patients with recurrent exacerbations with near normal lung function. Because of a bias in the patient recruitment process, there are significantly more patients from one site with a history of smoking and associated with cluster T2, so much so that the clinical variables for centre and for smoking status were confounded. We chose not to adjust the p-values for the centre effect as it would remove part or all of the variability associated with the smoking status.

Our phenotypes are quite distinguishable in terms of controlled versus uncontrolled asthma (Phenotype 1 versus Phenotypes 2, 3 and 4), airflow obstruction versus normal lung function (Phenotypes 2 and 3 versus Phenotypes 1 and 4), and infrequent exacerbations versus frequent exacerbations (Phenotypes 1, 2 and 3 versus Phenotype 4). Thus, our robust approach to clustering on the basis of clinico-physiological parameters has yielded phenotypes characterised on the basis of asthma control, airflow obstruction, recurrent exacerbations and oral corticosteroid dependence, which are all well-known features of severe asthma. The 3 clusters of predominantly severe asthma (clusters 2, 3 and 4) had the highest incidence of nasal polyps and exacerbations including admission to intensive care unit in the past year compared to Cluster 1. In addition, these 3 clusters also had the greatest use of rescue inhalers, of oral corticosteroid, and despite this, also had the higher ACQ5 scores. The differential expression of proteins and genes measured in sputum has also provided some insight into the potential pathophysiological pathways that may govern these phenotypes, particularly those related to the characteristics of severe asthma namely chronic airflow obstruction and frequent exacerbations.

In contrast to the training set, clustering of the validation set resulted in one additional cluster, even though the results were not as stable to resampling as in the training set, as shown by the cumulative distribution function and the deviation from ideal stability index. A potential reason for this may relate to the fact that we had fewer participants in the validation set compared to the training set thus increasing the difficulty of finding stable (to resampling) clusters. Furthermore, FEV1 (% predicted) and oral corticosteroid doses, two of the variables included in the clustering were slightly different between the training and validation sets. Nevertheless, the training and validation sets clusters shared similarities; cluster 4 being divided in the validation set into 2 clusters. This provides an internal replication of the clusters in our cohort..

Our clusters exhibit similarities with some of the clusters previously reported in two similar cohorts with mild/moderate and severe asthmatics, namely the SARP4 and the Leicester cohorts3, even though they used different clustering algorithms (Ward hierarchical clustering and k-means, respectively). In relation to the SARP cohorts, Phenotype 1 relates to SARP cluster 2, Phenotype 3 to SARP clusters 4 and 5, and Phenotype 4 to SARP cluster 3. One rather unique feature of our study is the inclusion of a smoking or ex-smoking cohort of severe asthma patients that were grouped principally in a late onset, severe airflow obstruction cluster with high blood and eosinophil counts and 55% of the group with evidence of atopy. These patients represent a group of asthma with features of COPD, namely chronic airflow obstruction, fulfilling the criteria of the asthma-COPD overlap syndrome5. A similar cluster has been previously reported27, 28, although in one cluster of smoking asthmatics, the degree of airflow obstruction was minimal but the cohort that was studied was not one of severe asthma29.

In the Leicester study3, the sputum eosinophil count was also used in the clustering, generating the late-onset, obese female severe asthmatics cluster with low sputum eosinophil counts, which is similar to Phenotype 4. In our clusters, blood and sputum eosinophil counts varied within each of these clusters with the highest to be found in the smoking and ex-smoking patients in Phenotype 2, supporting further the concept of the asthma-COPD overlap syndrome5. However, the clinical clusters did not segregate according to levels of serum IgE or FeNO. Furthermore, the levels of sputum periostin used as a biomarker of Th2-associated protein did not differ amongst the 4 groups.

These 4 distinct phenotypes of asthma that would be recognisable by the clinician experienced in seeing patients with severe asthma would allow the patients to be segregated into these clinical characteristics associated with severe asthma. These clusters were also characterised by different pathobiological pathways. Using very strict criteria for defining the differentially-abundant proteins by examining for consistency of their expression in both the training and validation sets analysed separately, we found that IL-16 (lymphocyte chemoattractant factor) was the only protein to be detected as differentially abundant when comparing both severe asthma clusters with airflow obstruction (present in Phenotypes 2 and 3) to well-controlled asthmatics (Phenotype 1). IL-16 has been previously associated with asthma, and shown to be expressed in abundance in epithelial cells after histamine challenge, in bronchoalveolar lavage fluid after an allergen challenge, in airway epithelium and CD4+ T-cells of airway biopsies30-32. Phenotype 2 showed higher levels than Phenotype 1 of CTAP III (CXCL7) and GM-CSF, which might be a reflection of the effect of smoking exposure, since CXCL7 has been used as a biomarker for the risk of lung cancer, and since GM-CSF mediates cigarette smoke-induced lung neutrophilia33, 34. In addition, higher levels of CXCL7 and GM-CSF has been shown in chronic obstructive pulmonary disease (COPD) secondary to cigarette smoking35, 36. On the other hand, Phenotype 3 showed decreased sputum levels of cathepsin G compared to Phenotype 1 and to Phenotype 4, in which raised systemic levels have been linked to neutrophilic asthma6.

LYN kinase was found to be reduced in Phenotype 3 (non-smoking severe obstructed asthmatics) when compared to Phenotype 2 (smoking or ex-smoking asthmatics). LYN kinase is a SRC kinase that controls GATA-3 and induces Th2 cell differentiation37 as well as the susceptibility of epithelial cells to their response to cigarette smoke extracts38. It has also been implicated in increasing asthma severity in mouse asthma models39.

Comparing the gene expression between Phenotypes 2 and 3 revealed pathways related to the regulation of actin cytoskeleton, and to fibronectin matrix formation. The comparison of Phenotype 4 (obese and exacerbation-prone asthmatics) with Phenotype 3 (airflow obstructed asthmatics), by contrast, yielded differential gene pathways related to immune cytokine signalling, particularly interferon signalling and regulation of fibroblast growth factor (FGF) and the signalling of FGF receptor (FGFR). These specific pathways may be involved in important pathophysiological aspects underlying the clinical phenotypes identified through this clustering approach based initially on clinico-physiological features.

Some of the limitations and biases within this analysis need to be highlighted. First, as in any clinical study, the cohort is biased by its inclusion and exclusion criteria (as discussed in detail in reference8) but we have been as inclusive as possible. Secondly, cluster analysis is a descriptive method and groups can be defined even when there is no underlying structure in the data; this limitation was addressed by assessing stability, separation and reproducibility of the clusters. Moreover, the choice of clinical variables may condition the type of clusters that is found but the choice of variables we used can be justified by their relevance to day-to-day clinical practice. The proof that the choice was reasonable is in the description of clinical cohorts that makes sense to the clinician. Finally, unsupervised clustering on the basis of the transcriptomic and proteomic data remains another powerful approach towards molecular phenotyping, work which is currently being performed in UBIOPRED.

**Conclusion**

The four phenotypes of asthma that we describe from the U-BIOPRED cohort have distinct clinical and molecular characteristics that should prove useful to the clinician in directing management of the particularly severe asthma phenotypes. One phenotype is associated with smoking, emphasising its influence on asthma. The differential molecular characteristics of the four phenotypes are not only potentially useful biomarkers of asthma severity, but they also represent a starting point for drug discovery efforts and the development of better treatments. This will pave the way towards a more personalised approach to asthma management.

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**Table I: Characteristics of the 4 asthma clusters from the training set□**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Variables** | **Missing or uncertain****T1 / T2 / T3 / T4** | **Cluster T1 (n = 69)** (moderate to severe, well controlled) | **Cluster T2 (n = 56)** (severe late onset asthma with airway obstruction, high BMI, smoking and OCS use) | **Cluster T 3 (n = 68)** (severe asthma with airway obstruction, OCS use but no smoking history) | **Cluster T4 (n = 73)** (severe asthma with female predominance, high BMI, frequent exacerbations, OCS use but no history of smoking or airway obstruction) | **P-Value** |
| Age (years) |  | 42.9 ± 15.6 | 57.4 ± 10.1 | 52.5 ± 15 | 47.5 ± 13.6 | **< 0.001 1** |
| Female gender |  | 55.07% | 57.14% | 47.06% | **83.56%** | **< 0.001 3** |
| Total daily OCS dose (normalised to milligrams prednisolone)† |  | 0 (0 – 0) | 0 (0 – 10) | **4 (0 – 10)** | 0 (0 – 10) | **< 0.001 2** |
| Asthma onset (years)† |  | 17 (5 – 30) | **42.5 (30.8 – 52)** | 17.5 (5.75 – 37) | 20 (7 – 37) | **< 0.001 2** |
| FEV1% predicted† |  | **88.5 ± 16.9** | 58.9 ± 15.6 | 48.5 ± 13.8 | **79.2 ± 15.4** | **< 0.001 1** |
| FEV1/FVC† |  | 0.737 ± 0.0836 | **0.557 ± 0.0978** | **0.505 ± 0.0787** | 0.741 ± 0.0832 | **< 0.001 1** |
| ACQ\_5† |  | **0.8 (0.25 – 1.6)** | 2 (1.2 – 2.8) | 2.4 (1.6 – 3.6) | 2.6 (1.8 – 3.2) | **< 0.001 2** |
| Number of exacerbations in past year† |  | 0 (0 – 1) | 1 (0.75 – 3) | 2 (1 – 3.25) | **3 (2 – 4)** | **< 0.001 2** |
| BMI (kg/m²)† |  | 25.1 (21.8 – 28.4) | 29 (26 – 33.3) | 25.4 (23.4 – 28.5) | **31.6 (26.8 – 35.8)** | **< 0.001 2** |
| Pack years† |  | 0 (0 – 0) | **16 (4.94 – 25.9)** | 0 (0 – 0) | 0 (0 – 0) | **< 0.001 2** |
| High ICS dose | **2 / 5 / 2 / 5** | **37.3%** | 98.0% | 96.9% | 95.5% | **< 0.001 3** |
| Blood eosinophils (x103/µl) | 0 / 1 / 1 / 4 | 0.199 (0.1 – 0.3) | **0.301 (0.119 – 0.56)** | 0.22 (0.1 – 0.494) | 0.2 (0.0997 – 0.385) | **0.0273 2** |
| Blood neutrophils (x103/µl) | **0 / 1 / 1 / 4** | **3.98 (3.10 – 4.87)** | 5.09 (3.97 – 7.15)NA's: 1 | 5.13 (3.74 – 7.61)NA's: 1 | 4.73 (3.62 – 6.70)NA's: 4 | **< 0.001 2** |
| Sputum eosinophils (%) | **39 / 20 / 44 / 45** | **0.78 (0.252 – 5.88)** | 4.88 (1.42 – 20)NA's: 20 | 3.67 (1.01 – 29.6)NA's: 44 | 2.42 (0.288 – 7.06)NA's: 45 | **0.0147 2** |
| Sputum neutrophils (%) | 39 / 20 / 44 / 45 | 54.4 (27.4 – 64.1) | 59.7 (44.0 – 70.5) | 63.4 (40.2 – 86.3) | 49.4 (31.5 – 70.0) | 0.268 2 |
| FeNO (ppb) | 1 / 1 / 0 / 10 | 24 (14 – 42.9) | 29 (15.5 – 53) | 33.8 (21.2 – 56.5) | 26.5 (15.5 – 48) | 0.332 2 |
| Total IgE (iu/ml) | 0 / 1 / 2 / 3 | 131 (54 – 316) | 140 (47 – 310) | 113 (41.5 – 352) | 124 (41.2 – 319)  | 0.995 2 |
| Atopy (positive) SPT or specific IgE | 0 / 2 / 0 / 1 | 84.1% | **55.6%** | 70.6% | 73.6% | **0.006 3** |

□ Values are presented as mean ± SD, median (1st – 3rd quartiles) or percentages.

1 ANOVA

2 Kruskal-Wallis test

3 χ² test

† Variables included in the clustering

**Table II:** **Differentially abundant proteins in sputum supernatants between clusters in a subset of patients**.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Target Name** | **GENE Symbol** | **Phenotype 2 vs 1****(n = 28 vs 22)** | **Phenotype 3 vs 1****(n = 20 vs 22)** | **Phenotype 3 vs 2****(n = 20 vs 28)** | **Phenotype 4 vs 3****(n = 16 vs 20)** |
| log2(FC)3 | P-value1, 2 | log2(FC)3 | P-value1, 2 | log2(FC)3 | P-value1, 2 | log2(FC)3 | P-value1, 2 |
| **IL-16** | **IL16** | 0.968 | **9.87e-6 (4.18e-3)** | 1.034 | **2.04e-5 (0.023)** | 0.066 |  | -0.153 |  |
| **CTAP-III** | **PPBP** | 1.096 | **4.61e-4 (9.13e-3)** | 1.141 |  | 0.045 |  | -0.432 |  |
| **Trypsin 2** | **PRSS2** | 0.930 | **1.85e-4 (6.72e-3)** | 0.833 |  | -0.097 |  | -0.283 |  |
| **GM-CSF** | **CSF2** | 0.898 | **6.69e-4 (0.0111)** | 0.242 |  | -0.656 |  | 0.0358 |  |
| **HPLN1** | **HAPLN1** | - 0.571 | **6.68e-5 (5.39e-3)** | -0.414 |  | 0.157 |  | -0.027 |  |
| **Cathepsin G** | **CTSG** | -0.682 |  | - 2.088 | **3.91e-5 (0.0221)** | -1.406 |  | 1.556 | 2.29e-3 (0.590) |
| **ARSB** | **ARSB** | 0.417 |  | 0.966 | 9.53e-4 (0.131) | 0.549 |  | -0.548 |  |
| **PSA2** | **PSMA2** | 0.140 |  | 0.955 | 7.82e-4 (0.126) | 0.815 |  | -0.456 |  |
| **LYN** | **LYN** | 1.038 |  | -1.338 |  | - 2.376 | **4.32e-5 (0.0488)** | 1.9002 |  |
| **FUT5** | **FUT5** | 0.283 |  | -1.821 |  | - 2.104 | 6.96e-4 (0.0982) | 1.537 |  |

1 Tukey p-value adjusted for age and gender in the training and validation set pooled together of the proteins that were consistently found both in the training and the validation for that specific contrast.

2 P-values are shown as Nominal (FDR corrected). Bold indicates that the difference is still significant even when correcting for false discovery rate using Benjamini-Hochberg method.

3 Fold change is given in base 2 logarithm, if it is positive in X vs Y it means that the analyte is more abundant in X than Y, negative values mean less abundant.

**Table III: Enriched pathways1 with differentially expressed genes between clusters in a subset of patients.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Term** | **Source** | **Size** | **Phenotype 2 vs 12,3****(n = 24 vs 31)** | **Phenotype 3 vs 12,3****(n = 20 vs 31)** | **Phenotype 3 vs 22,3****(n = 20 vs 24)** | **Phenotype 4 vs 32,3****(n = 19 vs 20)** |
| Hematopoietic cell lineage | KEGG | 84 | 0.05 (2) |  |  |  |
| Antigen processing and presentation | KEGG | 69 |  | 0.05 (5) |  |  |
| Regulation of actin cytoskeleton | KEGG | 216 |  |  | 0.05 (6) |  |
| Arrhythmogenic right ventricular cardiomyopathy  | KEGG | 74 |  |  | 0.032 (4) |  |
| Melanoma | KEGG | 72 |  |  |  | 0.032 (2) |
| Glioma | KEGG | 66 |  |  |  | 0.027 (2) |
| Non-small cell lung cancer | KEGG | 57 |  |  |  | 0.020 (2) |
| MicroRNAs in cancer | KEGG | 258 |  |  |  | 0.024 (3) |
| Prostate cancer | KEGG | 90 |  |  |  | 0.05 (2) |
| Pancreatic cancer | KEGG | 67 |  |  |  | 0.028 (2) |
| Bladder cancer | KEGG | 38 |  |  |  | 0.009 (2) |
| Chronic myeloid leukemia | KEGG | 74 |  |  |  | 0.034 (2) |
| N-glycan trimming in the ER and Calnexin/Calreticulin cycle | REAC | 13 |  | 0.05 (3) |  |  |
| Fibronectin matrix formation | REAC | 74 |  |  | 0.05 (2) |  |
| Negative regulation of FGFR signaling | REAC | 16 |  |  |  | 0.002 (2) |
| Spry regulation of FGF signaling | REAC | 16 |  |  |  | 0.002 (2) |
| Oncogene Induced Senescence | REAC | 31 |  |  |  | 0.010 (2) |
| Pre-NOTCH Expression and Processing | REAC | 57 |  |  |  | 0.035 (2) |
| Cytokine Signaling in Immune system | REAC | 309 |  |  |  | 0.005 (4) |
| Interferon Signaling | REAC | 194 |  |  |  | 0.022(3) |
| Interferon gamma signaling | REAC | 91 |  |  |  | 0.002 (3) |
| Interferon alpha/beta signaling | REAC | 68 |  |  |  | 0.05 (2) |

1 Pathways from KEGG and REACTOME databases.

2 P-values are calculated using hypergeometric test with FDR correction for multiple testing, as described in Reimand *et.al.*20.

3 Number of genes from the list found in the pathway (hits) is indicated in brackets. Only pathways with 2 hits or more are shown.

**Legend to Figures:**

**Figure 1: Clustering using PAM algorithm on the training set.**

Panel A represents the consensus Cumulative Distribution Function (CDF) of the consensus matrix shown in panel B. Panel C represents 2 internal quality indexes: the deviation from ideal stability and the Calinski and Harabasz index. Panel D shows a heat map of the pairwise distances between participants.

**Figure 2: Boxplots of main variables**

Boxplots of some variables used for clustering (A-F) together with oral corticosteroid (OCS) use as binary variable (I), blood eosinophil counts (G) and sputum eosinophil percentage (H). Clusters found in the training and validation sets exhibit similar distributions (light and dark hue of the same colour). The V4a and V4b clusters relate to 2 subsets of T4.

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