**‘T2-high’ in severe asthma related to blood eosinophil,**

**exhaled nitric oxide and serum periostin**

Stelios Pavlidis 1,2, Kentaro Takahashi 1,3, Francois Ng Kee Kwong 1, Jiaxing Xie 1, Uruj Hoda 1, Kai Sun2, Vahid Elyasigomari2, Paul Agapow2, Matthew Loza4, Fred Baribaud4, Pascal Chanez5, Steve J Fowler6, Dominic E. Shaw7, Louise J. Fleming1, Peter H. Howarth8, Ana R Sousa 9, Julie Corfield10, Charles Auffray11, Bertrand De Meulder11, Richard Knowles 12, Peter J Sterk13, Yike Guo2, Ian M. Adcock1,2, Ratko Djukanovic8, Kian Fan Chung1,2 *on behalf of the U-BIOPRED study group#*

1National Heart & Lung Institute, Imperial College London, & Biomedical Research Unit, Royal Brompton & Harefield NHS Trust, London, United Kingdom;

2Department of Computing & Data Science Institute, Imperial College London, United Kingdom;

3Research Centre for Allergy and Clinical Immunology, Asahi General Hospital, Asahi, Japan;

4Janssen Research and Development, High Wycombe, Buckinghamshire, United Kingdom;

5Assistance Publique des Hôpitaux de Marseille - Clinique des bronches, allergies et sommeil, Aix Marseille Université, Marseille, France

6Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom

7 Respiratory Research Unit, University of Nottingham, UK;

8 NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK

9Respiratory Therapeutic Unit, GSK, Stockley Park, UK;

10AstraZeneca R&D Molndal, Sweden and Areteva R&D, Nottingham, UK;

11 European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France

12 Knowles Consulting, Stevenage, UK

13Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;

#Consortium project team members are listed under *Acknowledgements*

**Word count:3400**

**Short title: ‘T-2 high’ in severe asthma**

**Key words:** Airway epithelial cells, IL-13, gene signature, exhaled NO, blood esoinophils.

**Corresponding author**

Professor K F Chung,

National Heart & Lung Institute,

Imperial College London,

Dovehouse Street,

London SW3 6LY, UK

Email: f.chung@imperial.ac.uk

**Summary: 199 words**

**Background**

Type-2 (T2) immune responses in airway epithelial cells (AECs) classifies mild-moderate asthma into a T2-high phenotype. We examined whether currently-available clinical biomarkers can predict AEC-defined T2-high phenotype within U-BOPRED cohort.

**Methods**

The transcriptomic profile of AECs obtained from brushings of 103 patients with asthma and 44healthy controls was obtained and gene set variation analysis used to determine the relative expression score of T2 asthma using a signature from IL-13-exposed AECs.

**Results**

37% of asthmatics (45% non-smoking severe asthma, n=49, 33% of smoking or ex-smoking severe asthma, n=18 and 28% mild-moderate asthma, n=36) were T2-high using AEC gene expression. They were more symptomatic with higher levels of nitric oxide in exhaled breath (FeNO) and of blood and sputum eosinophils but not of serum IgE or periostin. Sputum eosinophilia correlated best with the T2-high signature. FeNO (≥30 ppb) and blood eosinophils (≥300/µL) gave a moderate prediction of T2-high asthma. Sputum IL-4, IL-5 and IL-13 protein levels did not correlate with gene expression.

**Conclusion**

T2-high severe asthma can be predicted to some extent from raised levels of FeNO, blood and sputum eosinophil counts, but serum IgE or serum periostin were poor predictors. Better bedside biomarkers are needed to detect T2-high.

**Key words:** Type 2 asthma, serum periostin, nitric oxide, blood eosinophil, sputum eosinophil, severe asthma, bronchial brushings, airway epithelium

**Take home message:**

T2-high was found in 45% non-smoking and 33% of smoking or ex-smoking severe asthma, and 28% mild-moderate asthma. This can be predicted from raised levels of nitric oxide in exhaled breath, blood and sputum eosinophil counts but not from serum periostin.

**Introduction**

Asthma, particularly severe asthma, is a heterogeneous disease in terms of its clinical presentation and inflammatory components (1). Cluster analysis of severe asthma on the basis of clinico-physiologic parameters have delineated several phenotypes characterised by age of onset of disease, chronic airflow obstruction, recurrent exacerbations and eosinophilic inflammation (2-4). However, the mechanisms underlying these different phenotypes remain unclear. In mild-to-moderate asthma, using the expression of 3 genes, periostin *(POSTN)*, chloride channel accessory 1 *(CLCA1)* and Serpin β2 *(SERPINB2),* that are overexpressed in airway epithelial cells when stimulated by the Type 2 (T2) cytokine IL-13, a ‘T2-high’ phenotype has been defined. The T2-high phenotype was found to be associated with high blood eosinophil count, a greater expression of IL-5 in bronchial biopsies, more severe bronchial responsiveness and a beneficial response to inhaled corticosteroids when compared to those with ‘T2-low’(5).

There is little information regarding patients with severe asthma as to the expression of ‘T2-high’ phenotype. We have therefore examined the levels of the T2-high asthma signature in patients with severe asthma from the U-BIOPRED cohort and determined the reliability of currently available biomarkers such as blood eosinophil count, exhaled breath nitric oxide (FeNO) levels and serum periostin in predicting an epithelial T2-high phenotype. The latter is becoming an important issue to address because of the introduction of antibody-based biologic treatments targeting T2 cytokines such as anti-IL5, anti-IL5Rα and anti-IL4Rα antibody therapies for severe asthma, and the use of these currently-available biomarkers to select responders to these treatments (6-8).

**Methods**

**Study design**

U-BIOPRED is a European multicentre prospective cohort study that recruited severe non-smoking asthma (SAn), smokers and ex-smokers with severe asthma (SAs), mild/moderate non-smoking asthmatics (MMA) and healthy non-smoking controls (HC) (9). Because the definition of T2-high has relied on the analysis of the transcriptome of epithelial brushings, we chose the subjects who underwent a fiberoptic bronchoscopic procedure for obtaining bronchial brushings. The characteristics of these study participants are shown in Table 1. We analysed transcriptomic data from 49 SAn, 18 SAs, 36 MMA and 44 HC. Pre-bronchodilator spirometry,

FeNO, serum total IgE, serum periostin and differential blood count were measured as previously described (9). The study was approved by the Ethics Committees of the recruiting centres and all participants gave written informed consent.

**Protein and other assays**

The SOMAscan proteomic assay of sputum supernatants performed by SomaLogic Inc., (Boulder, CO) was used to obtain 1129 analytes (10). Serum periostin was measured using a proprietary sandwich ELISA with 2 mAbs capable of detecting all known splice variants of human periostin (11). Serum or plasma cytokines IL-1α, IL-13, IL-17A, CCL11, CCL18 and, CCL26 were assayed by ELISA techniques. FeNO was measured with an electrochemical analyser (NIOX MINO; Aerocrine AB, Solna, Sweden) at an expiratory flow rate of 50 ml s−1. Serum IgE was measured by Thermo Fisher Pharmacia CAP system.

**Microarray analysis of epithelial brushings transcriptome**

Expression profiling of epithelial brushings was performed using Affymetrix HT HG-U133+ PM microarray platform (Affymetrix, Santa Clara, Calif). RNA purity (RIN >9.5) was measured by Agilent Bioanalyser (Agilent, Santa Clara, Calif). Cell pellets from epithelial brushings were stored in RNA stabilization buffer (Norgen Biotek, Thorhold, Canada). RNA purity (RIN >6) was measured by Agilent Bioanalyser (Agilent, Santa Clara, Calif). Raw data were quality-assessed and pre-processed by robust multi-array average normalization using the Almac Pipeline and Pre-processing Toolbox (Almac, Craigavon, United Kingdom). False discovery rate (FDR) using the Benjamini and Hochberg method was applied for p-value adjustment in relation to multiple tests.

**T2 signatures summarized by gene set variation analysis (GSVA)**

We evaluated gene expression related to a T2 immune response following two separate analytical approaches. First, we utilised Gene Set Variation Analysis (GSVA) which calculates sample-wise enrichment scores (ES) irrespective of any group labels (12). This unsupervised approach allowed us to observe the variation in the activity of a set of genes over an entire sample population. By annotating each subject using summarization of the genes related to T2, we compared the cumulative expression of T2-related gene sets between patient groups. For this purpose, we used a T2 gene set consisting of 34 genes that were upregulated following *in vitro* stimulation of airway epithelial lung cells with IL-13 (IL13 IVS) in experiments carried out in the laboratories of Janssen Research and Development (13).

Second, we examined the expression of 3 T2 gene biomarkers (*POSTN, SERPINB2, CLCA1*) previously proposed by Woodruff et al (5, 14) that was derived from airway epithelial cells of patients with asthma. Because the expression of *CLCA1* was found below levels of quality detection, we utilised only the normalised average expression of *POSTN* and *SERPINB2* to identify T2-high samples.

**Statistical analysis**

All datasets for this analysis were curated and stored in tranSMART, an open-source knowledge management platform for sharing research data (15), supported by the European Translational Information and Knowledge Management Services (eTRIKS) project [https://www.etriks.org/, 2017]. All categorical variables were analysed using Fisher’s exact test. ANOVA was used for continuous variables with normal distribution, while Wilcoxon rank-sum test was used for variables with skewed distribution. For establishing the significance of GSVA results, we used a linear model for differential gene expression analysis, implemented in the limma R Bioconductor package (16) to analyse the ES differences between groups.

**RESULTS**

**Clinical features of cohort**

We analysed the distribution of clinical features and biomarkers in those who have had bronchial epithelial brushings performed (**Table 1**). Atopy was higher in the SAn group and serum IgE particularly in the smoking SAs group. Blood and sputum eosinophils were significantly higher in asthma patients, while FEV1 was lower, particularly in the severe asthma groups. FeNO was increased in SAn group but was lower in SAs compared to MMA.

**T2-high and T2-low**

We applied gene set variation analysis to the bronchial brushings gene expression data, using the J&J IL13 IVS signature (Fold change>2, FDR<0.05) *(13)*. The 95th percentile of enrichment of this signature in the healthy population was utilised as a threshold to obtain T2-high and T2-low subjects (**Fig 1A**). We found that 45% of the severe asthma non-smokers (22 out of 49) and 33% of the smoking severe asthmatics (6 out of 18) were classified as T2-high. In the mild/moderate asthmatics, 28% (10 out of 36) patients were classified as T2-high (**Fig 1A**).

The average normalised expression of the previously-proposed markers of T2-high asthma, *POSTN, SERPINB2* and *CLCA1* (5), was also determined. Because CLCA1 gene expression was below the lower level of detection in the bronchial brushing microarray data, we only examined *POSTN* and *SERPINB2* and defined the 95th of their normalised average expression in the healthy controls as threshold for identifying T2-high subjects (**Fig 1B**). This analysis produced a lower proportion of patients being classified as T2-high in each cohort, as compared with the IL13 IVS signature (**Fig 1C**) (A: 17/49 or 35%, B: 4/18 or 22%, C: 5/36 or 14%). A comparison of the two classification approaches revealed moderate agreement, with Cohen’s kappa value of agreement equal to 0.59 (p =10-8). Patients classified as T2-high based on the expression of *POSTN* and *SERPINB2* were found to be a subset of those classified as such by the IL13 IVS gene signature (**Fig 1C**), indicating that the former approach was more conservative.

**Clinical features of T2 high and T2-low asthma**

We analysed the U-BIOPRED clinical features of the T2-low and T2-high groups, as defined by the T2 IL-13 IVS gene signature (**Table 2**). T2-high patients as compared to T2-low patients exhibited higher levels of symptoms, showed a greater bronchodilator response to salbutamol and was composed of a higher percentage of patients on oral corticosteroid therapy.

The levels of the biomarkers, FeNO, blood and sputum eosinophils, serum IgE and serum periostin were examined in T2-high and T2-low groups (**Fig 2**). Except for serum periostin levels, the levels of these biomarkers were significantly elevated in the asthmatic groups compared to healthy subjects. Although there was a wide overlap in the levels of these biomarkers between T2-high and T2-low groups, the levels of most biomarkers exhibited a significant increase in the T2-high compared to the T2-low group, with the exception of serum IgE and serum periostin.

**Predicting T2-high from clinical biomarkers**

Receiver-operating characteristics (ROC) curve analysis was performed to examine how well these biomarkers can predict the T2-high/low classification, using the IL-13 IVS gene signature (**Fig 3**). This analysis was implemented with the pROC R package which applies bootstrapping to produce the curves and estimate confidence intervals. The area under the ROC curve (AUC) was 67.8% for FeNO, 68.9% for blood eosinophil counts, 78.1% for sputum eosinophils (%), 62.2% for serum IgE, levels and 55.6% for serum periostin, indicating a poor to fair prediction using FeNO, blood eosinophils and sputum eosinophils and serum IgE, but with no predictive value for serum periostin.

We then explored the association of the T2 biomarkers, FeNO, blood eosinophils and serum periostin, with the enrichment score of the IL-13 IVS signature. FeNO≥30ppb, blood eosinophil counts ≥ 300/µl and serum periostin ≥55µg/l were defined as threshold to consider the biomarkers as abnormally high. **Figure 4A** displays a Venn Diagram showing the overlap of patients with high T2 biomarkers values and the average enrichment score of the IL-13 IVS signature in each patient group. Looking at each biomarker separately, the T2 ES score was high in patients with high FeNO (ES=0.13) as well as in patients with high blood eosinophil count (ES=0.13), but less so in patients with high levels of serum periostin (ES=0.05). The highest ES was observed in patients with both high FeNO and blood eosinophils, while serum periostin did not perform well. When all three biomarkers were examined together, the ES score of the IL-13 IVS signature was slightly reduced. The ES score of each individual patient, based on grouping according to the levels of each individual T2 biomarker and their combinations is shown in **Figure 4B**. The ES score of the T2 IL-13 IVS gene signature was significantly, but weakly, correlated with FeNO (r=0.29, p-value=10-3) and blood eosinophil counts (r=0.34, p-value=10-6), but not with serum periostin levels (r=-0.04, p-value=0.64). The T2 IL-13 IVS ES score was strongly correlated with the sputum eosinophil count (r =0.55, p-value=10-6).

We also applied chi-square test to determine whether there is any contingency between T2-high and T2-low status defined by the IL-13 IVS signature and high and low defined levels of the biomarkers FeNO (≥ or < 30ppb) and blood eosinophil counts (≥ or < 300/µl), and of the combination of FeNO and blood eosinophil count (**Table 3**). All p-values were significant (p-value<0.05) rejecting the null hypothesis that the eosinophil and FeNO status of patients is not contingent with the T2 status. Serum periostin and the combination of periostin with FeNO or eosinophils did not reach significance.

**Serum and sputum IL-4, IL-5 and IL-13 levels**

We examined whether the levels of the T2 cytokines IL-4, IL-5 and IL-13 in sputum and serum could be useful in predicting the degree of T2 expression. In SOMAscan analysis of serum samples, levels of IL-4, IL-5 and IL-13 were similar across healthy, T2-high and T2-low patients (**Fig 5**). In sputum samples, IL-4 and IL-13 cytokine levels were also similar across all cohorts, but IL-5 concentrations were higher in severe asthma. IL-4 and IL-5 sputum levels were negatively correlated (r=-0.61, p-value = 10-12), while IL-4 and IL-13 were positively correlated (r=0.42, p-value = 10-6). We found no correlation between the gene expression of *POSTN, IL5, IL4, and IL13* in bronchial brushings and the concentration of the corresponding proteins in sputum. Serum IL-13 and CCL18 and plasma CCL11 and CCL26 levels were increased in T2-high, in addition to serum IL-1α (**Table 2**).

**CCL26 gene expression in the epithelium as a marker of T2-high asthma**

 The recent ADEPT study (13) reported that CCL26 gene expression, the highest gene expressed in the IL-13 IVS signature, was enhanced in moderate/severe asthma and was a good predictor of T2-high asthma. We, therefore, analysed CCL26 gene expression in relation to T2 expression in the airway epithelium. While CCL26 levels were generally very low (mean CCL26 log2 intensity = 3.29), there were 28 patients with CCL26 expression that were above the 95th percentile of the distribution in the healthy population in 28 patients, including 22 severe asthmatics and 6 mild/moderate patients (**Supplemental Figure 1A**). The expression of CCL26 was significantly higher in the T2-high group as defined by the enrichment of the IL-13 IVS signature (**Supplemental Figure 1B**). Using CCL26 expression levels to segregate patients into T2-high/low, produced groups with similar T2 biomarker distributions as with the IL-13 IVS signature based distribution. FeNO and sputum eosinophils were significantly higher in the T2-high group, compared to T2-low and healthy (**Supplemental Figure 2**). In fact, the CCL26 and IL-13 IVS T2 high asthma definition approaches showed some overlap, in terms of the patients’ selection (**Supplemental Figure 3**). CCL26 expression in patients with high FeNO, blood eosinophils and periostin were similar (Fig 3A). CCL26 expression was highest in patients with high FeNO and blood eosinophils (**Supplemental Figure 4**). Only FeNO high (≥ 30ppb) patients, but not those with high blood eosinophil counts, showed a significant association with the CCL26 T2-high defined status (**Supplemental Table 1**).

**Discussion**

We determined the ‘T2-high’ and ‘T2-low’ phenotype in patients with asthma by measuring the expression of a 34-gene signature derived from the genes that were overexpressed in airway epithelial cells exposed to IL-13 using the method of GSVA (12). Using the cut-off point for ‘T2-high’ as being above the 95th centile of the expression scores in our non-asthmatic controls, we found that 37% of asthmatic participants (with 45% non-smoking severe asthma, 33% of smoking or ex-smoking severe asthma and 28% mild-moderate asthma) were T2-high. Thus, the severe non-smoking asthma had the highest expression of T2-high, higher than the mild-moderate asthma. The clinical phenotype of the T2-high patients as compared to the T2-low patients was found to be predominantly male and their asthma control was poorer as assessed by a higher ACQ5 score. In addition, these patients also demonstrated a higher bronchodilator response and were slightly more obstructed with an increased total lung capacity. The extended IL-13 IVIS signature defined a larger number of Th2 severe asthmatic patients than that defined by the 2-gene *POSTN*  and *SERPINB2* expression.

T2-high patients had a higher total serum IgE, FeNO, blood eosinophil and sputum eosinophil counts, but serum periostin levels did not differentiate between T2-high and T2-low asthmatics. Thus, in contrast to the clinical characteristics of the mild asthmatics defined as T2-high in the study of Woodruff et al (5), our T2-high moderate-to-severe asthma had features indicative of poorer control and more severe asthma, but both T2-high groups shared higher levels of serum IgE, FeNO and blood eosinophil counts. However, ROC curve analysis showed that FeNO and blood and sputum eosinophil counts were poor predictors of T2-high in severe asthma. The combination of high FeNO and high blood eosinophil counts achieved the highest agreement with the T2-high classification derived from the 34-gene IL-13 IVS signature from IL-13-activated airway epithelial cells (13). However, this combination only provided marginal improvement over the use of either biomarker singly and the predictive value remained low.

We compared the application of the IL-13 IVS gene signature with the 3 genes (periostin, CLCA1 and Serpin B2) previously used to define T2-high in a group of mild asthmatics that showed that 50% of such patients had a T2 high signature (5). Because one of the 3 genes, *CLCA1*, was hardly expressed in the epithelial cells of our asthmatic subjects, we focused on the expression of *SERPINB2* and *periostin* genes only. We found a lower proportion of patients were classified as T2-high compared to the application of the IL-13 IVS signature, such that the use of the 2-gene signature providing a more conservative result. Only 25.2% of patients could be classified as T2-high using the 2-gene signature. Interestingly, in the mild-to-moderate group, we found a very low percentage of patients as being T2-high (5 out of 36 at 14%) which is much lower than the 50% of T2-high previously reported in mild asthmatic subjects (5). By contrast, using the IL-13 IVS signature, the T2-high percentage was 28% (10 out of 36) in the same mild-to-moderate group, indicating that this signature was more comprehensive.

Other approaches have been previously used to define T2-high asthma. Thus, gene expression of T2-associated cytokines, IL4, IL5 and IL13, has been measured in induced sputum cells by RT-PCR (17). Using a combined metric of gene expression of these 3 cytokines, Peters et al have reported that 70% of 37 asthmatics had T2-high asthma. However, the levels of these transcripts detected in sputum were extremely low and, as reported in a previous study, we were unable to detect the expression of these genes by either Affymetrix chip assay or by 40 cycles of PCR in sputum cell samples of patients with severe asthma (18). On the other hand, we detected measurable levels of the proteins IL-4, IL5 and IL-13 in sputum supernatants and in serum samples by using the high throughput SOMAscan protein assay platform that use single-stranded DNA-based protein affinity reagents to assay proteins (19). However, the levels of these cytokines in sputum or serum did not distinguish T2 high from T2-low. On the contrary, direct assays of the chemokines CCL-18, CCL11 and CCL26 which are involved in T2-inflammation and eosinophil biology were significantly elevated in the serum in T2-high asthmatic patients.

A composite biomarker composed of FeNO, blood eosinophil count and serum periostin has been used as marker of T2-high, and a high level of this composite score has been shown to differentiate subjects with severe asthma as having a high risk of exacerbations (20). However, we did not find that serum periostin levels had any predictive value for T2-high asthma. This is in agreement with a study that showed that serum periostin was unable to distinguish eosinophilic from non-eosinophilic airway inflammation measured in sputum from mild-to-moderate asthma subjects (21). This is the first time that the values of these bedside biomarkers in predicting T2-high status in patients with severe asthma has been examined. Currently, bedside biomarkers are being used to pinpoint patients with severe asthma who are suitable for, and will respond therapeutically to, targeted T2-therapies such as anti-IL5 or anti-IL5R antibody treatments. We examined the use of combined FeNO and blood eosinophil count as a predictor of high T2 using a model fit, but found only marginal improvement in the AUC of the ROC curve to 71.6% compared to 68.9% for blood eosinophil counts and 67.8% for FeNO. Therefore, there is a need to develop better bedside biomarkers to pinpoint asthma patients with T2-high, particularly those that respond to these targeted therapies (22).

In the ADEPT study of moderate-to-severe asthma, it was reported that all subjects with high airway mucosal CC26 gene expression in the epithelium was associated with high FeNO levels and high blood eosinophil counts, compared to only 36% of subjects with low mucosal levels of CCL26 gene expression (13). However, in our patients, we did not find such discrimination in that those with high mucosal level of CCL26 could be associated with either high or low levels of FeNO or blood eosinophil counts (data not shown). This might be explained by the fact that the severe asthma patients in ADEPT were not as severe as those in U-BIOPRED, as illustrated by only 1 out of 51 severe asthma patients compared to 45% of the U-BIOPRED severe asthma cohort being on regular oral corticosteroid therapy (23). This raises the likelihood of the suppressive effects of systemic corticosteroid therapy on these serum biomarkers interfering with their predictive properties.

In summary, we found that the majority of patients with severe asthma fall into the T2-low category when the T2 status is defined by the expression of the IL13 IVS gene signature. There is a reasonable predictive value of high FeNO and high blood eosinophil count in predicting T2-high status, with a combination of high FeNO and high blood eosinophil count being marginally a better predictor. Although a high sputum eosinophil count provides the best predictor of T2 high asthma, as defined by airway epithelial expression profiling, this was only modest. Better bedside predictor biomarkers for T2-high are therefore needed.

**Acknowledgements:**

The U-BIOPRED project is supported through an Innovative Medicines Initiative joint undertaking under grant agreement 115010, resources of which are composed of financial contributions from the European Union’s Seventh Framework Programme (FP7/2007-2013) and European Federation of Pharmaceutical Industries and Associations companies’ in-kind contributions (www.imi.europa.eu).

**Table 1. Characteristics of study participants.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Cohort** | **SAn (N = 49)** | **SAs (N = 18)** | **MMA (N = 36)** | **Healthy (N = 44)** | **p-value** |
| Age (Mean, SD) [N] | 48.6 (14.36) [49]  | 51.9 (8.35) [18] | 38.3 (13.70) [36] | 37.6 (13.77) [44] | 10-5 |
| Female (N/%) [N] | 25 (51%) [49] | 7 (39%) [18] | 21 (58%) [36] | 16 (36%) [44] | 0.19 |
| Atopy (N/%) [N] | 35 (71%) [48] | 12 (67%) [18] | 27 (75%) [36] | 14 (32%) [44] | 0.0001 |
| Total IgE IU/ML (Mean, SD) [N] | 318.113 (445.6) [49] | 623.594 (1565.6) [17] | 281.024 (701.7) [36] | 64.005 (104.7) [42] | 0.041 |
| On Oral corticosteroids (N/%) [N] | 19 (38.8%) [45] | 9 (50.0%) [17] | 0 [36] | NA | 0.589 |
| FEV1 % (Mean, SD) [N] | 75.8 (21.6) [49] | 66.2 (14.9) [18] | 93.2 (16.7) [36] | 101.1 (11.9) [44] | 10-14 |
| FeNO ppb (Mean, SD) [N] | 41.77 (29.49) [45] | 27.68 (25.15) [17] | 36.74 (33.78) [36] | 21.89 (14.49) [42] | 0.005 |
| Blood Eosinophils /μL (Mean, SD) [N] | 288.8 (238.1) [49] | 264.4 (268.4) [18] | 231.1 (164.7) [36] | 157 .0 (146.3) [44] | 0.018 |
| Sputum Eosinophils % (Mean, SD) [N] | 12.275 (20.29) [21] | 11.718 (17.7090) [7] | 2.373 (3.8992) [15] | 0.313 (0.47) [25] | 0.028 |
| BMI (Mean, SD) [N] | 30.6 (6.3) [49] | 29.1 (6.2) [18] | 26.4 (4.7) [36] | 24.8 (3.3) [44] | 10-6 |
| Exacerbations (Mean, SD) [N] | 2.5 (2.0) [48] | 2.3 (1.7) [18] | 0.36 (0.79) [36] | NA | 10-7 |
| ACQ5 (Mean, SD) [N] | 1.9 (1.1) [39] | 2.3 (1.2) [13] | 0.9 (0.7) [35] | NA | 10-6 |
| Nasal Polyps (N/%) [N] | 18 (40.1%) [44] | 7 (41.2%) [17] | 2 (5.8%) [34] | 3 (21%) [14] | 0.003 |

ACQ5: Asthma Control Questionnaire with 5 questions; BMI: Body Mass index; FeNO: Level of nitric oxide in exhaled breath; FEV1: Forced expiratory volume in one second; IgE: Immunoglobulin E; MMA: Mild/Moderate non-smoking asthmatics; ppb: parts per billion; SAn: Severe non-smoking asthma; SAs: Smokers and ex-smokers with severe asthma.

**Table 2. Characteristics of study participants as T2-high and T2-low**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **T2-high** | **T2-low** | p-value |
| Age (Mean, SD) [N] | 46.8 (15.1) [38] | 45.2 (13.6) [65] | 0.52 |
| Female (N/%)[N] | 13 (38.0%) [38] | 40 (61.5%) [65] | 0.01 |
| BMI (Mean, SD) [N] | 27.6 (5.6) [38] | 29.7(6.2) [65] | 0.11 |
| Atopy (N/%)[N] | 27 (84.4%) [32] | 47 (78.3%) [60] | 0.67 |
| Total IgE IU/mL (Median, IQR) [N] | 171 (316.5) [38] | 85.2 (243.9) [64] | 0.04 |
| On oral corticosteroids (N/%)[N] | 15 (40.5%) [37] | 13 (21.3%) [61] | 0.069 |
| FEV1 % predicted (Mean, SD) [N] | 75.4 (21.9) [38] | 83.0 (20.7) [65] | 0.06 |
| FEV1/FVC (%) (Mean, SD) [N] | 65.3 (11.9) [37] | 69.6 (11.3) [65] | 0.11 |
| FVC % predicted (Mean, SD) [N] | 94.1 (18.5) [38] | 98.4 (18.3) [65] | 0.13 |
| FEV1 % increase post-salbutamol (Mean, SD) [N] | 15.4 (14.3) [37] | 9.6 (10.0) [64] | 0.02 |
| FeNO ppb (Mean, SD) [N] | 50.4 (35.1) [36] | 29.9 (25.1) [62] | 0.003 |
| Exacerbations (Mean, SD) [N] | 2.1(2.3) [38] | 1.4(1.6) [64] | 0.24 |
| Nasal Polyps diagnosed (N/%)[N] | 11(31.4%) [35] | 16(26.6%) [60] | 0.79 |
| ACQ5 (Mean, SD) [N] | 1.9 (0.9) [33] | 1.4(1.1) [54] | 0.01 |
| Blood Eosinophils /mL (Median, IQR) [N] | 285 (215) [38] | 200 (200) [65] | 0.001 |
| Blood Neutrophils /mL | 4370 (3565) [38] | 4100 (2300) [65] | 0.59 |
| Sputum Eosinophils % (Median, IQR) [N] | 4.64 (19.1) [19] | 0.48 (1.1) [25] | 0.001 |
| Sputum Neutrophils % (Median, IQR) [N] | 52.3 (29.9) [12] | 49.8 (33.9) [15] | 0.44 |
| Sputum Lymphocytes % (Median, IQR) [N] | 0.92 (1.0) [19] | 1.29 (1.1) [24] | 0.034 |
| Sputum Macrophages % (Median, IQR) [N] | 27.6 (32.9) [19] | 45.1 (30.0) [24] | 0.016 |
| Serum CCL18 pg/ml (Median, IQR) [N] | 170.3 (73.7) [33] | 127.2 (96.2) [56] | 0.03 |
| Serum IL17A pg/ml (Median, IQR) [N] | 0.28 (0.17) [33] | 0.32 (0.25) [55] | 0.17 |
| Serum periostin ng/ml (Median, IQR) [N] | 46.7 (19.2) [33] | 45.4 (12.0) [55] | 0.38 |
| Serum IL-1α pg/ml (Median, IQR) [N] | 36.2 (9.6) [38] | 32.9 (9.6) [64] | 0.009 |
| Plasma CCL11 pg/ml (Median, IQR) [N] | 106.26 (40.83) [35] | 94.82 (57.40) [58] | 0.02 |
| Plasma CCL26 (Median, IQR) [N] | 23.3 (15.8) [35] | 13.9 (16.9) [58] | 0.002 |

ACQ5: Asthma Control Questionnaire with 5 questions; BMI: Body Mass index; FeNO: Level of nitric oxide in exhaled breath; FEV1: Forced expiratory volume in one second; FVC: Forced expiratory volume; IgE: Immunoglobulin E; MMA: Mild/Moderate non-smoking asthmatics; ppb: parts per billion; SAn: Severe non-smoking asthma; SAs: Smokers and ex-smokers with severe asthma.

**Table 3. Contingency tables for biomarker cut-off points with T2-high and T2-low asthma defined by the IL-13 IVS signature**

|  |  |  |  |
| --- | --- | --- | --- |
| **Group** | **T2 high** | **T2 low** | **P value** |
| FeNO ≥30ppb | 23 | 19 | 0.001 |
| FeNO <30ppb | 12 | 44 |
|  |  |  |  |
| Blood Eos ≥300/μl | 19 | 18 | 0.026 |
| Blood Eos <300/μl | 18 | 48 |
|  |  |  |  |
| Periostin ≥55μg/L | 9 | 12 | 0.59 |
| Periostin <55μg/L | 23 | 46 |
|  |  |  |  |
| Eos ≥300/μl & FeNO ≥30ppb | 13 | 6 | 0.002 |
| Eos <300/μl & FeNO <30ppb | 22 | 57 |
|  |  |  |  |
| Eos ≥300/μl & Periostin ≥55μg/L | 7 | 6 | 0.24 |
| Eos <300/μ & Periostin <55μg/L | 28 | 57 |
|  |  |  |  |
| FeNO ≥30ppb & Periostin ≥55μg/L | 7 | 4 | 0.24 |
| FeNO <30ppb & Periostin <55μg/L | 28 | 59 |
|  |  |  |  |
| Eos ≥300/μl & FeNO ≥30ppb& Periostin ≥55μg/L | 6 | 2 | 0.02 |
| Eos <300/μl & FeNO <30ppb & Periostin <55μg/L | 29 | 61 |

**Legend to Figures:**

**Fig 1.** **T2-high transcriptomic-based definition.** A. Enrichment scores (ES) of participants for a gene signature (IL-13 IVS T2 signature) consisting of genes up-regulated following *in-vitro* stimulation of bronchial epithelial cells with IL-13 in the U-BIOPRED clinical groups. The 95th percentile of the ES score in healthy controls (HC) was estimated at 0.129 and used to classify the samples into T2-high and T2-Low. B. Average normalised expression of *POSTN* and *SERPINB2* genes in the U-BIOPRED clinical groups. The 95th percentile in HC, estimated at log2 intensity of 1.701, was used to classify asthma patients into T2-high and T2-low. C. Venn diagrams showing T2 classification agreement between the GSVA IL-13 gene signature methodand the *POSTN* and *SERPINB2* approach in all patients, and in the 3 separate asthma groups. There is close agreement between the two methods, with 88 patients classified in the same categories and disagreement for 18 out of 103, with Cohen's Kappa score = 0.63. HC: Healthy controls; MMA: Mild/Moderate non-smoking asthmatics; ppb: parts per billion; SAn: Severe non-smoking asthma; SAs: Smokers and ex-smokers with severe asthma.

**Fig 2**. **Box plots of T2 biomarkers distribution in healthy controls (HC) and patients classified as T2-low and T2-high.** A. Classification based on the enrichment score (ES) of the IL-13 T2-high gene signature. B. Classification based on the normalized average expression of *POSTN* and *SERPINB*2 genes. FeNO: Level of nitric oxide in exhaled breath; IgE: Immunoglobulin E. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.

**Fig 3.** **Receiver Operating Characteristic curves for classification of asthmatic patients as T2-high, based on FeNO, blood eosinophils and serum periostin.** T2 categorization was based on IL-13 T2 IVS signature. Receiver operating characteristics (ROC) analysis of peripheral blood eosinophils and FeNO and serum periostin as biomarkers of T2 expression was performed, using the pROC R package which applies bootstrapping to produce the curves and estimate confidence intervals. For FeNO, the highest sensitivity and specificity were observed at a threshold of 41.5 ppb (AUC= 67.8%). For peripheral blood eosinophil count, the highest combination of sensitivity and specificity was reached at 115/µL (AUC=68.9%). For sputum eosinophils, the highest combination of sensitivity and specificity was reached at 1.4% (AUC=78.1%). For serum IgE, the highest combination of sensitivity and specificity was reached at 124 IU/mL (AUC=62.2%). Periostin exhibited the worse performance, achieving classification equivalent to random.

**Fig 4.** A. Venn Diagram displaying overlap of patients with high levels of FeNO (≥ 30ppb), blood eosinophils (≥300/µL), and serum periostin (≥55µg/L). B. Box plot of IL-13 T2-high signature enrichment score (ES) distribution, based on the cut-off levels of FeNO, serum periostin (PER) and blood eosinophil counts (EOS), as shown in the Venn diagrams in panel A. In addition, ES scores are shown for combination of the cut-off points.

**Fig 5.** Box plot of Somascan measured levels of the T2-cytokines, IL-4, IL-5 and IL-13, in serum and sputum supernatants of healthy controls (HC), and T2-low and T2-high asthmatic patients. T2 high and low groups were defined based on the IL-13 IVS signature. There were no significant differences between any of the groups. RFU: Relative Fluorescent Units.

**References**

1. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. Eur Respir J. 2014;43(2):343-73.

2. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. Am J Respir Crit Care Med. 2010;181(4):315-23.

3. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al. Cluster analysis and clinical asthma phenotypes. AmJRespir Crit Care Med. 2008;178(3):218-24.

4. Lefaudeux D, De Meulder B, Loza MJ, Peffer N, Rowe A, Baribaud F, et al. U-BIOPRED clinical adult asthma clusters linked to a subset of sputum omics. J Allergy Clin Immunol. 2016.

5. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. AmJRespir Crit Care Med. 2009;180(5):388-95.

6. Ortega HG, Liu MC, Pavord ID, Brusselle GG, FitzGerald JM, Chetta A, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. N Engl J Med. 2014;371(13):1198-207.

7. Castro M, Wenzel SE, Bleecker ER, Pizzichini E, Kuna P, Busse WW, et al. Benralizumab, an anti-interleukin 5 receptor alpha monoclonal antibody, versus placebo for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study. The lancet Respiratory medicine. 2014;2(11):879-90.

8. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, et al. Lebrikizumab treatment in adults with asthma. N Engl J Med. 2011;365(12):1088-98.

9. Shaw DE, Sousa AR, Fowler SJ, Fleming LJ, Roberts G, Corfield J, et al. Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort. Eur Respir J. 2015;46(5):1308-21.

10. Kuo CS, Pavlidis S, Loza M, Baribaud F, Rowe A, Pandis I, et al. T-helper cell type 2 (Th2) and non-Th2 molecular phenotypes of asthma using sputum transcriptomics in U-BIOPRED. Eur Respir J. 2017;49(2).

11. Jia G, Erickson RW, Choy DF, Mosesova S, Wu LC, Solberg OD, et al. Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. J Allergy Clin Immunol. 2012;130(3):647-54.e10.

12. Hanzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. BMC bioinformatics. 2013;14:7.

13. Silkoff PE, Laviolette M, Singh D, FitzGerald JM, Kelsen S, Backer V, et al. Identification of airway mucosal type 2 inflammation by using clinical biomarkers in asthmatic patients. J Allergy Clin Immunol. 2017;140(3):710-9.

14. Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proc Natl Acad Sci U S A. 2007;104(40):15858-63.

15. Athey BD, Braxenthaler M, Haas M, Guo Y. tranSMART: An Open Source and Community-Driven Informatics and Data Sharing Platform for Clinical and Translational Research. AMIA Joint Summits on Translational Science proceedings AMIA Summit on Translational Science. 2013;2013:6-8.

16. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47.

17. Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV. Measures of gene expression in sputum cells can identify T2-high and T2-low subtypes of asthma. J Allergy Clin Immunol. 2013.

18. Rossios C, Pavlidis S, Hoda U, Kuo CH, Wiegman C, Russell K, et al. Sputum transcriptomics reveal up-regulation of IL-1 receptor family members in severe asthma. J Allergy Clin Immunol. 2017.

19. Rohloff JC, Gelinas AD, Jarvis TC, Ochsner UA, Schneider DJ, Gold L, et al. Nucleic Acid Ligands With Protein-like Side Chains: Modified Aptamers and Their Use as Diagnostic and Therapeutic Agents. Molecular therapy Nucleic acids. 2014;3:e201.

20. Heaney LG, Djukanovic R, Woodcock A, Walker S, Matthews JG, Pavord ID, et al. Research in progress: Medical Research Council United Kingdom Refractory Asthma Stratification Programme (RASP-UK). Thorax. 2016;71(2):187-9.

21. Wagener AH, de Nijs SB, Lutter R, Sousa AR, Weersink EJ, Bel EH, et al. External validation of blood eosinophils, FE(NO) and serum periostin as surrogates for sputum eosinophils in asthma. Thorax. 2015;70(2):115-20.

22. Chung KF. Asthma phenotyping: a necessity for improved therapeutic precision and new targeted therapies. J Intern Med. 2016;279(2):192-204.

23. Silkoff PE, Strambu I, Laviolette M, Singh D, FitzGerald JM, Lam S, et al. Asthma characteristics and biomarkers from the Airways Disease Endotyping for Personalized Therapeutics (ADEPT) longitudinal profiling study. Respiratory research. 2015;16:142.