"T2-high" in severe asthma related to blood eosinophil, exhaled nitric oxide and serum periostin

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T2-high was found in 45% of nonsmoking and 33% of smoking/ex-smoking severe asthma and 28% of 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. http://ow.ly/Zsq630myR9t

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ABSTRACT 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 the U-BIOPRED cohort.

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

37% of asthmatics (45% nonsmoking 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 exhaled nitric oxide fraction (F_{ENO}) and blood and sputum eosinophils, but not serum IgE or periostin. Sputum eosinophilia correlated best with the T2-high signature. F_{ENO} (⩾30 ppb) and blood eosinophils (⩾300 cells·μL^{-1}) gave a moderate prediction of T2-high asthma. Sputum IL-4, IL-5 and IL-13 protein levels did not correlate with gene expression.

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

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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-physiological 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–moderate asthma, using the expression of three genes, periostin (*POSTN*), chloride channel accessory 1 (*CLCA1*) and Serpin β2 (*SERPINB2*), which are overexpressed in airway epithelial cells when stimulated by the type 2 (T2) cytokine interleukin (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 as to the expression of the T2-high phenotype in patients with severe asthma. Therefore, we have 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 nitric oxide fraction (F_{ENO}) 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 biological treatments targeting T2 cytokines such as anti-IL-5, anti-IL-5Rα and anti-IL-4Rα 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 nonsmoking asthma, smokers and ex-smokers with severe asthma, mild–moderate nonsmoking asthmatics and healthy nonsmoking controls [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 fibre-optic bronchoscopic procedure for obtaining bronchial brushings. The characteristics of these study participants are shown in table 1. We analysed transcriptomic data from 49 severe nonsmoking asthma, 18 smokers and ex-smokers with severe asthma, 36 mild–moderate nonsmoking asthmatics and 44 healthy nonsmoking controls. Pre-bronchodilator spirometry, F_{ENO}, 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 (Boulder, CO, USA) was used to obtain 1129 analytes [10]. Serum periostin was measured using a proprietary sandwich ELISA with two monoclonal antibodies 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 using ELISA techniques. F_{ENO} was measured using an electrochemical analyser (NIOX MINO; Aerocrine, Solna, Sweden) at an expiratory flow rate of 50 $mL \cdot s^{-1}$. Serum IgE was measured using the Thermo Fisher (Uppsala, Sweden) 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, CA, USA). RNA purity (RNA integrity number (RIN) >9.5) was measured using Agilent Bioanalyser (Agilent, Santa Clara). Cell pellets from epithelial brushings were

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TABLE 1 Characteristics of study participants

	SAn	Subjects n	SAs	Subjects n	MMA	Subjects n	HC	Subjects n	p-value
Subjects n	49		18		36		33		
Age years	48.6±14.36	49	51.9±8.35	18	38.3±13.70	36	37.6±13.77	44	10^{-5}
Female n (%)	25 (51)	49	7 (39)	18	21 (58)	36	16 (36)	44	0.19
Atopy n (%)	35 (71)	48	12 (67)	18	27 (75)	36	14 (32)	44	10^{-4}
Total IgE IU·mL^{-1}	318.1±445.6	49	623.6±1565.6	17	281.0±701.7	36	64.0±104.7	42	0.041
On oral corticosteroids n (%)	19 (38.8)	45	9 (50.0)	17	0	36	NA		0.58
On inhaled corticosteroids n (%)	100%	49	100%	18	100%	36	NA		
FEV_1 % pred	74.5 (33.1)	49	69.5 (18.1)	18	95.1 (21.4)	36	100.7 (13.3)	44	10^{-14}
FEV_1/FVC	78.8 (3.6)	49	78.7 (2.3)	18	81.8 (4.3)	36	82.3 (3.4)	44	0.004
FVC % pred	90.5 (25.4)	49	92.9 (13.6)	18	109.7 (25.9)	36	110.3 (11.4)	44	10^{-4}
FEV_1 % increase post-salbutamol	7.14 (13.7)	49	13.7 (7.8)	18	8.2 (9.9)	34	NA		0.02
F_{ENO} ppb	32.5 (37.0)	45	20.0 (17.5)	17	21.0 (40.1)	36	16.3 (14.8)	42	0.005
Blood eosinophils cells·µL^{-1}	200.0 (300.0)	49	200.0 (52.5)	18	200.0 (200.0)	36	100 .0 (102.5)	44	0.018
Sputum eosinophils %	1.4 (15.4)	21	0.8 (12.9)	7	0.6 (2.7)	15	0.0 (0.2)	25	0.028
BMI kg·m^{-2}	30.6±6.3	49	29.1±6.2	18	26.4±4.7	36	24.8±3.3	44	10^{-6}
Exacerbations n	2.5±2.0	48	2.3±1.7	18	0.36±0.79	36	NA		10^{-7}
ACQ5	1.9±1.1	39	2.3±1.2	13	0.9±0.7	35	NA		10^{-6}
Nasal polyps n (%)	18 (40.1)	44	7 (41.2)	17	2 (5.8)	34	3 (21)	14	0.003

Data are presented as n, mean±SD or median (interquartile range), unless otherwise stated. SAn: severe nonsmoking asthma; SAs: smokers and ex-smokers with severe asthma; MMA: mild–moderate nonsmoking asthma; HC: healthy nonsmoking controls; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; F_{ENO}: exhaled nitric oxide fraction; BMI: body mass index; ACQ5: 5-item asthma control questionnaire; NA: not applicable.

stored in RNA stabilisation buffer (Norgen Biotek, Thorhold, ON, Canada). RNA purity (RIN >6) was measured using Agilent Bioanalyser. Raw data were quality-assessed and pre-processed using robust multi-array average normalisation using the Almac Pipeline and Pre-processing Toolbox (Almac, Craigavon, UK). False discovery rate (FDR) using the Benjamini and Hochberg method was applied for p-value adjustment in relation to multiple tests.

T2 signatures summarised by gene set variation analysis

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 summarisation 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 (IL-13 IVS) in experiments carried out in the laboratories of Janssen Research and Development [13].

Second, we examined the expression of three T2 gene biomarkers (*POSTN*, *SERPINB2*, *CLCA1*) previously proposed by Woodruff and co-workers [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 (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 the cohort

We analysed the distribution of clinical features and biomarkers in those who had bronchial epithelial brushings performed (table 1). Atopy was higher in the severe nonsmoking asthma group and serum IgE

was particularly high in the smoking group. Blood and sputum eosinophils were significantly higher in asthma patients, while forced expiratory volume in 1 s was lower, particularly in the severe asthma groups. *F*eNO was increased in the severe nonsmoking asthma group, but was lower in smokers and ex-smokers with severe asthma compared to mild–moderate nonsmoking asthma patients.

T2-high and T2-low

We applied GSVA to the bronchial brushings gene expression data, using the J&J IL-13 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 (figure 1a). We found that 45% of the severe asthma nonsmokers (22 out of 49), 50% of the currently smoking severe asthmatics (three out of six) and 25% of the ex-smoking severe asthmatics (three out of 12) were classified as T2-high. In the mild–moderate asthmatics, 28% (10 out of 36) patients were classified as T2-high (figure 1a).

In addition, the average normalised expression of the previously-proposed markers of T2-high asthma, *POSTN*, *SERPINB2* and *CLCA1* [5] was 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 percentile of their normalised average expression in the healthy controls as threshold for identifying T2-high subjects (figure 1b). This analysis produced a lower proportion of patients being classified as T2-high in each cohort, as compared with the IL-13 IVS signature: 17 (35%) out of 49 severe asthma nonsmokers; four (22%) out of 18 currently smoking severe asthmatics; and five (14%) out of 36 mild–moderate asthmatics (figure 1c). A comparison of the two classification approaches revealed moderate agreement, with Cohen's κ 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 IL-13 IVS gene signature (figure 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. All asthmatic patients were on inhaled corticosteroid therapy and there were no significant differences in terms of inhaled corticosteroid therapies between the T2-high and T2-low groups. Of the five patients on therapy with anti-IgE therapy, three were T2-low and two T2-high. In terms of comorbidities, there was a greater incidence of sinusitis, allergy and surgical treatment (supplementary table S1); conversely, there was a tendency for fewer gastro-oesophageal reflux disease symptoms and psychiatric problems.

The levels of the biomarkers, *F*eNO, blood and sputum eosinophils, serum IgE and serum periostin were examined in T2-high and T2-low groups (figure 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.

In addition, we examined the features of T2-high *versus* T2-low in the severe asthma patients alone (supplementary table S2) and showed that the T2-high severe asthma still had a higher blood eosinophil and higher sputum eosinophil count (%), although the latter was not statistically significant, associated with a lower sputum macrophage count (%) compared to T2-low severe asthma. Serum IL-1α was also significantly higher in T2-high.

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 (figure 3). This analysis was implemented with the pROC R package (www.r-project.org), which applies bootstrapping to produce the curves and estimate confidence intervals. The area under the ROC curve (AUC) was 67.8% for *F*eNO, 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 *F*eNO, 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, *F*eNO, blood eosinophils and serum periostin, with the enrichment score of the IL-13 IVS signature. *F*eNO ⩾30 ppb, blood eosinophil counts ⩾300 cells·μL^{-1} and serum periostin ⩾55 μg·L^{-1} were defined as threshold to consider the biomarkers as abnormally high. Figure 4a presents a Venn diagram showing the overlap of patients with high T2 biomarker values and the average enrichment score of the IL-13 IVS signature in each patient group. Looking at each biomarker

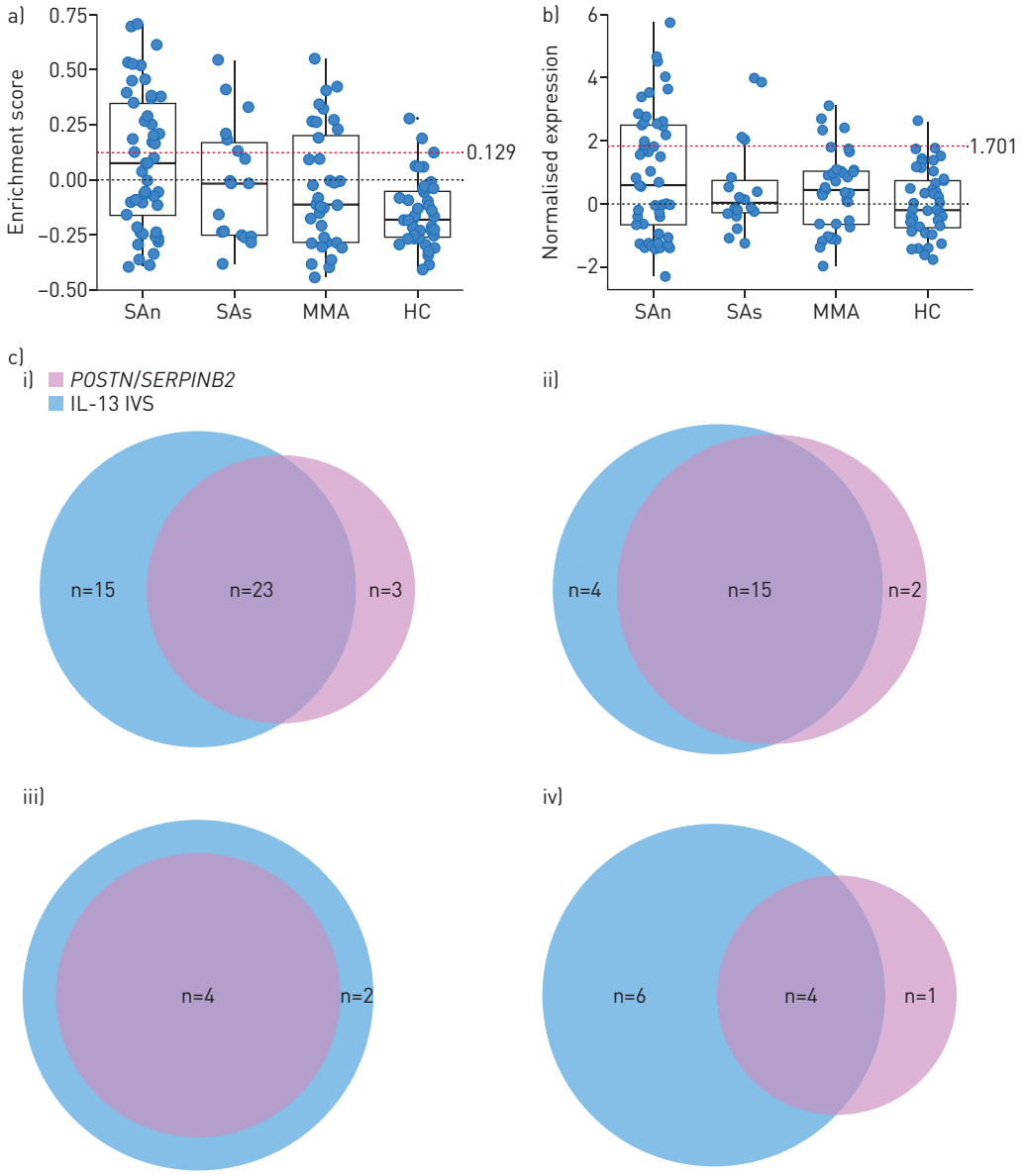

FIGURE 1 T2-high transcriptomic-based definition. a) Enrichment scores of participants for a gene signature (*in vitro* stimulation of airway epithelial lung cells with interleukin (IL)-13 (IL-13 IVS) T2 signature) consisting of genes upregulated following *in vitro* stimulation of bronchial epithelial cells with IL-13 in the U-BIOPRED clinical groups. The 95th percentile of the enrichment 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 $\log_2$ 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 gene set variation analysis IL-13 gene signature method and the *POSTN* and *SERPINB2* approach in i) all patients (n=103), and in the three separate asthma groups: ii) severe nonsmoking asthma (SAn) (n=49); iii) smokers and ex-smokers with severe asthma (SAs) (n=18); and iv) mild–moderate nonsmoking asthmatics (MMA) (n=36). 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 κ=0.63.

separately, the T2 enrichment score was high in patients with high F_{ENO} (0.13) as well as in patients with high blood eosinophil count (0.13), but less so in patients with high levels of serum periostin (0.05). The highest enrichment score was observed in patients with both high F_{ENO} and blood eosinophils, while serum periostin did not perform well. When all three biomarkers were examined together, the enrichment 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 enrichment score of the T2 IL-13 IVS gene signature was significantly, but weakly, correlated with F_{ENO} (r=0.29, $p=10^{-3}$) and blood eosinophil counts (r=0.34, $p=10^{-6}$), but not with serum

TABLE 2 Characteristics of study asthma participants as T2-high and T2-low

	T2-high	Subjects n	T2-low	Subjects n	p-value
Age years	46.8±15.1	38	45.2±13.6	65	0.52
Female n (%)	13 (38.0)	38	40 (61.5)	65	0.01
BMI kg·m^{-2}	27.6±5.6	38	29.7±6.2	65	0.11
Atopy n (%)	27 (84.4)	32	47 (78.3)	60	0.67
Total IgE IU·mL^{-1}	171 (316.5)	38	85.2 (243.9)	64	0.04
Current smokers n (%)	3 (7.9)	38	3 (4.6)	65	0.60
Ex-smokers n (%)	9 (23.7)	38	17 (26.2)	65	1.0
On inhaled corticosteroids n (%)	27 (71.1)	38	47 (75.8)	62	0.64
On oral corticosteroids n (%)	15 (40.5)	37	13 (21.3)	61	0.069
Anti IgE therapy n (%)	2 (5.2)	38	3 (4.6)	65	1.0
FEV_1 % predicted	71.2 (34.1)	38	88.7 (24.7)	65	0.06
FEV_1/FVC %	66.5 (21.5)	37	69.4 (16.6)	65	0.11
FVC % predicted	91.8 (16.6)	38	99.5 (28.7)	65	0.13
FEV_1 % increase post-salbutamol	12.34 (13.5)	37	7.3 (10.1)	64	0.02
F_{ENO} ppb	46.0 (45.5)	36	21.0 (19.0)	62	0.003
Exacerbations	1.5 (3.75)	38	1.0 (2)	64	0.24
Nasal polyps diagnosed n (%)	11 (31.4)	35	16 (26.6)	60	0.79
ACQ5	1.9±0.9	33	1.4±1.1	54	0.01
Blood eosinophils cells·μL^{-1}	285 (215)	38	200 (200)	65	0.001
Blood neutrophils cells·μL^{-1}	4370 (3565)	38	4100 (2300)	65	0.59
Sputum eosinophils %	4.64 (19.1)	19	0.48 (1.1)	25	0.001
Sputum neutrophils %	52.3 (29.9)	12	49.8 (33.9)	15	0.44
Sputum lymphocytes %	0.92 (1.0)	19	1.29 (1.1)	24	0.034
Sputum macrophages %	27.6 (32.9)	19	45.1 (30.0)	24	0.016
Serum CCL18 pg·mL^{-1}	170.3 (73.7)	33	127.2 (96.2)	56	0.03
Serum IL-17A pg·mL^{-1}	0.28 (0.17)	33	0.32 (0.25)	55	0.17
Serum periostin ng·mL^{-1}	46.7 (19.2)	33	45.4 (12.0)	55	0.38
Serum IL-1α pg·mL^{-1}	36.2 (9.6)	38	32.9 (9.6)	64	0.009
Plasma CCL11 pg·mL^{-1}	106.26 (40.83)	35	94.82 (57.40)	58	0.02
Plasma CCL26 pg·mL^{-1}	23.3 (15.8)	35	13.9 (16.9)	58	0.002

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; F_{ENO}: exhaled nitric oxide fraction; ACQ5: 5-item asthma control questionnaire; IL: interleukin.

periostin levels (r=−0.04, p=0.64). The T2 IL-13 IVS enrichment score was strongly correlated with the sputum eosinophil count (r=0.55, p=10^{-6}).

We also applied the Chi-squared 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 F_{ENO} (⩾ or <30 ppb) and blood eosinophil counts (⩾ or <300 cells·μL^{-1}), and of the combination of F_{ENO} and blood eosinophil count (table 3). All p-values were significant (p<0.05), rejecting the null hypothesis that the eosinophil and F_{ENO} status of patients is not contingent with T2 status. Serum periostin and the combination of periostin with F_{ENO} 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 (figure 5). Additionally, in sputum samples, IL-4 and IL-13 cytokine levels were 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=10^{-12}), while IL-4 and IL-13 were positively correlated (r=0.42, p=10^{-6}). We found no correlation between the gene expression of *POSTN*, *IL-5*, *IL-4*, and *IL-13* 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 individuals, in addition to serum IL-1α (table 2).

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

The recent Airways Disease Endotyping for Personalized Therapeutics (ADEPT) study [13] reported that CCL26 gene expression, the gene with the highest expression in the IL-13 IVS signature, was enhanced in

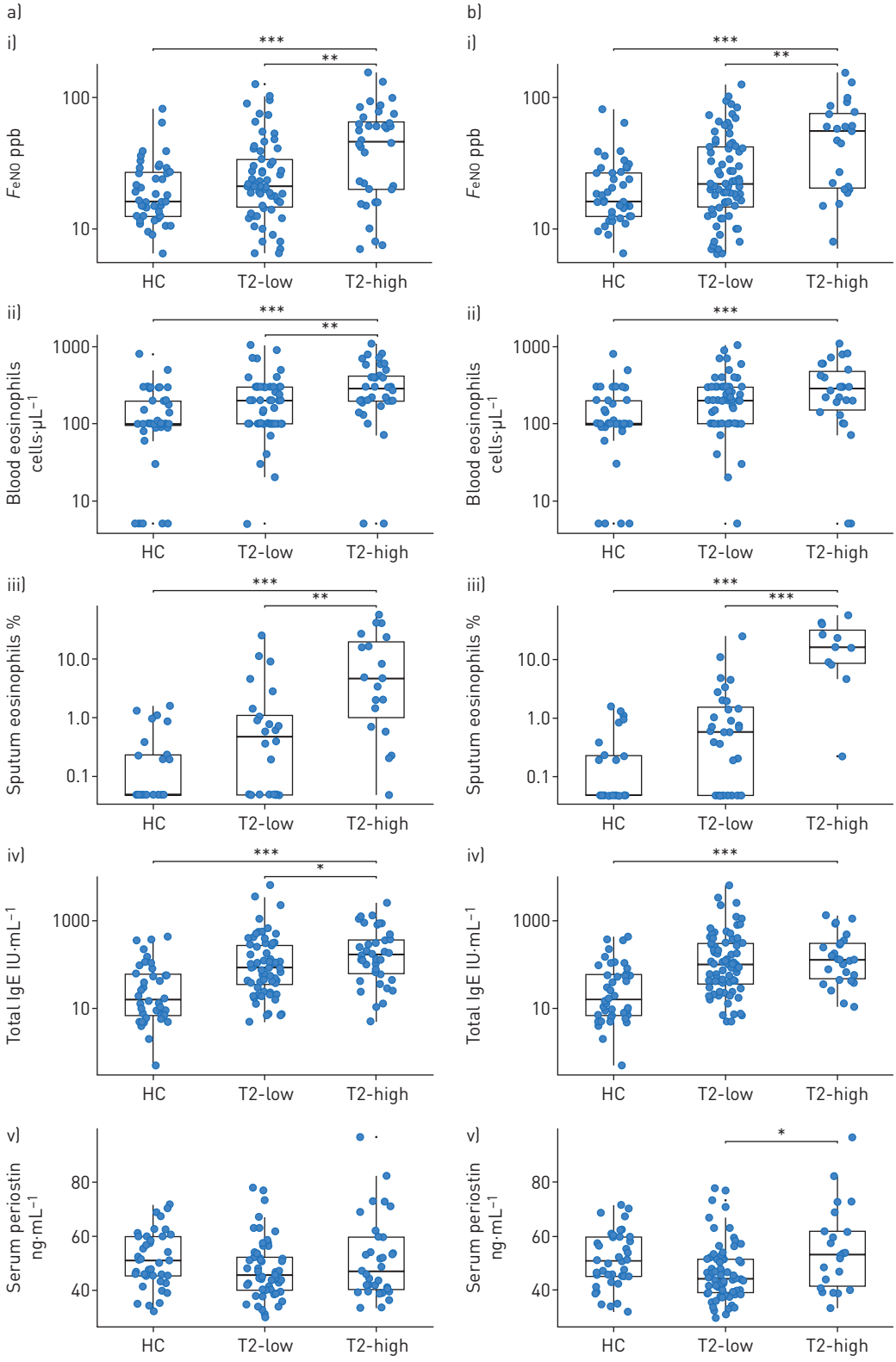

FIGURE 2 Box plots of T2 biomarkers distribution in healthy controls (HC) and patients classified as T2-low and T2-high. a) Classification based on the enrichment score (ES) of the interleukin (IL)-13 T2-high gene signature; b) classification based on the normalised average expression of *POSTN* and *SERPINB2* genes. F_{eNO}: exhaled nitric oxide fraction. *: p<0.05; **: p<0.01; ***: p<0.001.

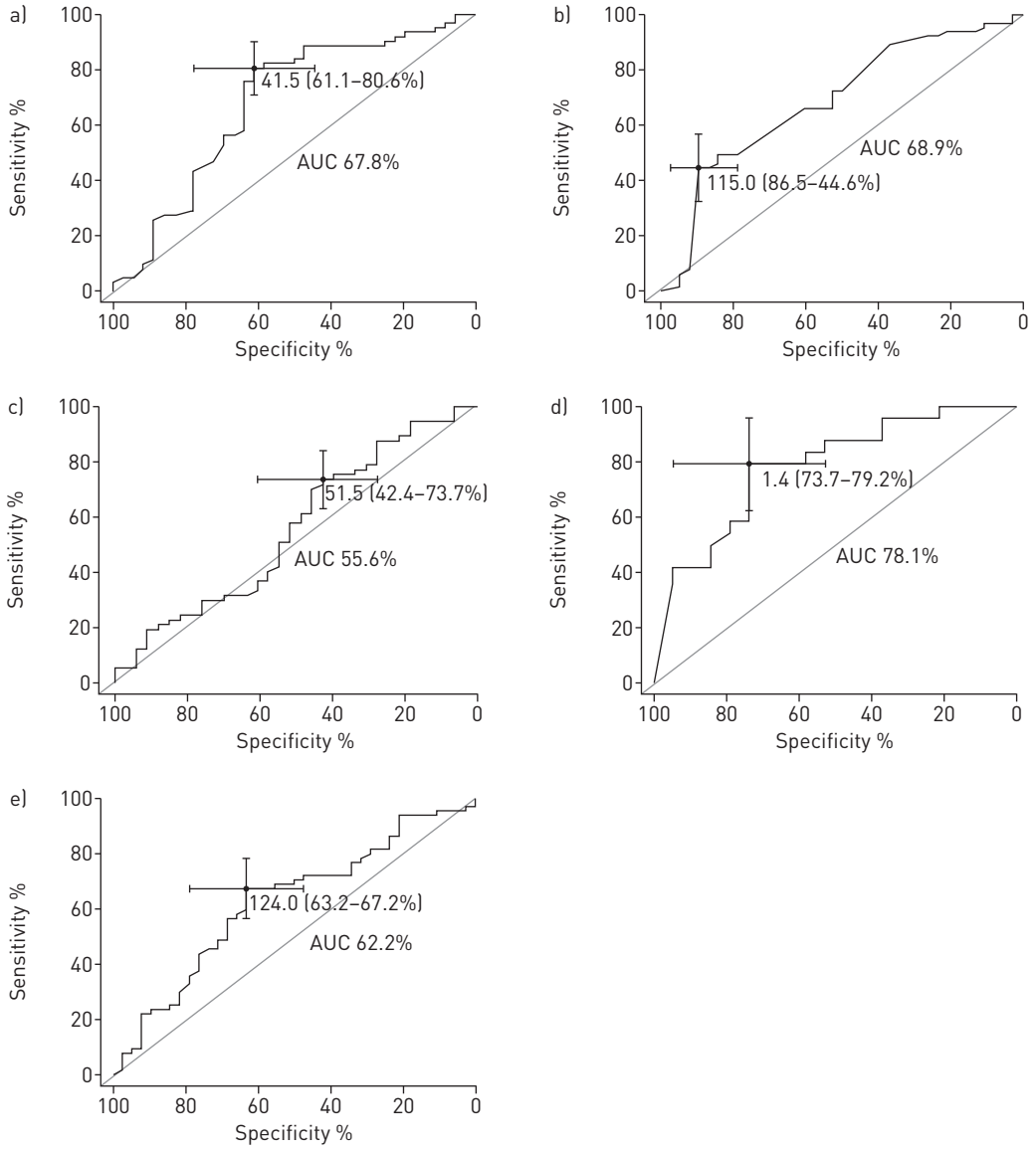

FIGURE 3 Receiver operating characteristic (ROC) curves for classification of asthmatic patients as T2-high, based on a) exhaled nitric oxide fraction (F_{ENO}); b) blood eosinophils; and c) serum periostin. T2 categorisation was based on *in vitro* stimulation of airway epithelial lung cells with interleukin (IL)-13 (IL-13 IVS) T2 signature. ROC analysis of peripheral blood eosinophils and F_{ENO} 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 F_{ENO}, the highest sensitivity and specificity were observed at a threshold of 41.5 ppb (area under the curve (AUC) 67.8%). For peripheral blood eosinophil count, the highest combination of sensitivity and specificity was reached at 115 cells·μL^{-1} (AUC 68.9%). d) For sputum eosinophils, the highest combination of sensitivity and specificity was reached at 1.4% (AUC 78.1%). e) For serum IgE, the highest combination of sensitivity and specificity was reached at 124 IU·mL^{-1} (AUC 62.2%). Periostin exhibited the worst performance, achieving classification equivalent to random.

moderate–severe asthma and was a good predictor of T2-high asthma. Therefore, we analysed CCL26 gene expression in relation to T2 expression in the airway epithelium. While CCL26 levels were generally very low (mean CCL26 $\log_2$ 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 six mild–moderate patients (supplementary figure S1a). The expression of CCL26 was significantly higher in the T2-high group as defined by the enrichment of the IL-13 IVS signature (supplementary figure S1b). 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. F_{ENO} and sputum eosinophils were significantly higher in the T2-high group compared to T2-low and healthy (supplementary figure S2). In fact, the CCL26 and IL-13 IVS T2-high asthma definition approaches

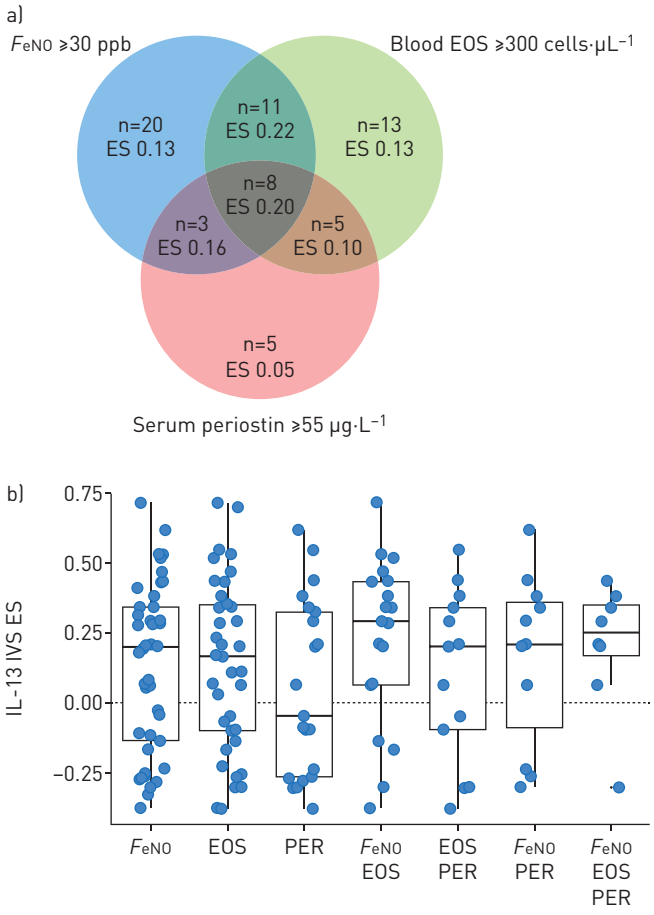

FIGURE 4 a) Venn diagram displaying overlap of patients with high levels of exhaled nitric oxide fraction (F_{ENO}) (⩾30 ppb), blood eosinophils (⩾300 cells·μL^{-1}) and serum periostin (⩾55 μg·L^{-1}); b) box plot of interleukin (IL)-13 T2-high signature enrichment score (ES) distribution, based on the cut-off levels of F_{ENO}, serum periostin (PER) and blood eosinophil counts (EOS), as shown in the Venn diagrams in a). In addition, ES scores are shown for a combination of the cut-off points.

TABLE 3 Contingency tables for biomarker cut-off points with T2-high and T2-low asthma defined by the *in vitro* stimulation of airway epithelial lung cells with interleukin (IL)-13 (IL-13 IVS) signature

	T2-high	T2-low	p-value
F_{ENO} ⩾30 ppb	23	19	0.001
F_{ENO} <30 ppb	12	44	
Blood eosinophils ⩾300 cells·μL^{-1}	19	18	0.026
Blood eosinophils <300 cells·μL^{-1}	18	48	
Serum periostin ⩾55 μg·L^{-1}	9	12	0.59
Serum periostin <55 μg·L^{-1}	23	46	
Blood eosinophils ⩾300 μg·L^{-1} and F_{ENO} ⩾30 ppb	13	6	0.002
Blood eosinophils <300 μg·L^{-1} and F_{ENO} <30 ppb	22	57	
Blood eosinophils ⩾300 μg·L^{-1} and serum periostin ⩾55 μg·L^{-1}	7	6	0.24
Blood eosinophils <300 μg·L^{-1} and serum periostin <55 μg·L^{-1}	28	57	
F_{ENO} ⩾30 ppb and serum periostin ⩾55 μg·L^{-1}	7	4	0.24
F_{ENO} <30 ppb and serum periostin <55 μg·L^{-1}	28	59	
Blood eosinophils ⩾300 μg·L^{-1} and F_{ENO} ⩾30 ppb and serum periostin ⩾55 μg·L^{-1}	6	2	0.02
Blood eosinophils <300 μg·L^{-1} and F_{ENO} <30 ppb and serum periostin <55 μg·L^{-1}	29	61	

Data are presented as n, unless otherwise stated. F_{ENO}: exhaled nitric oxide fraction.

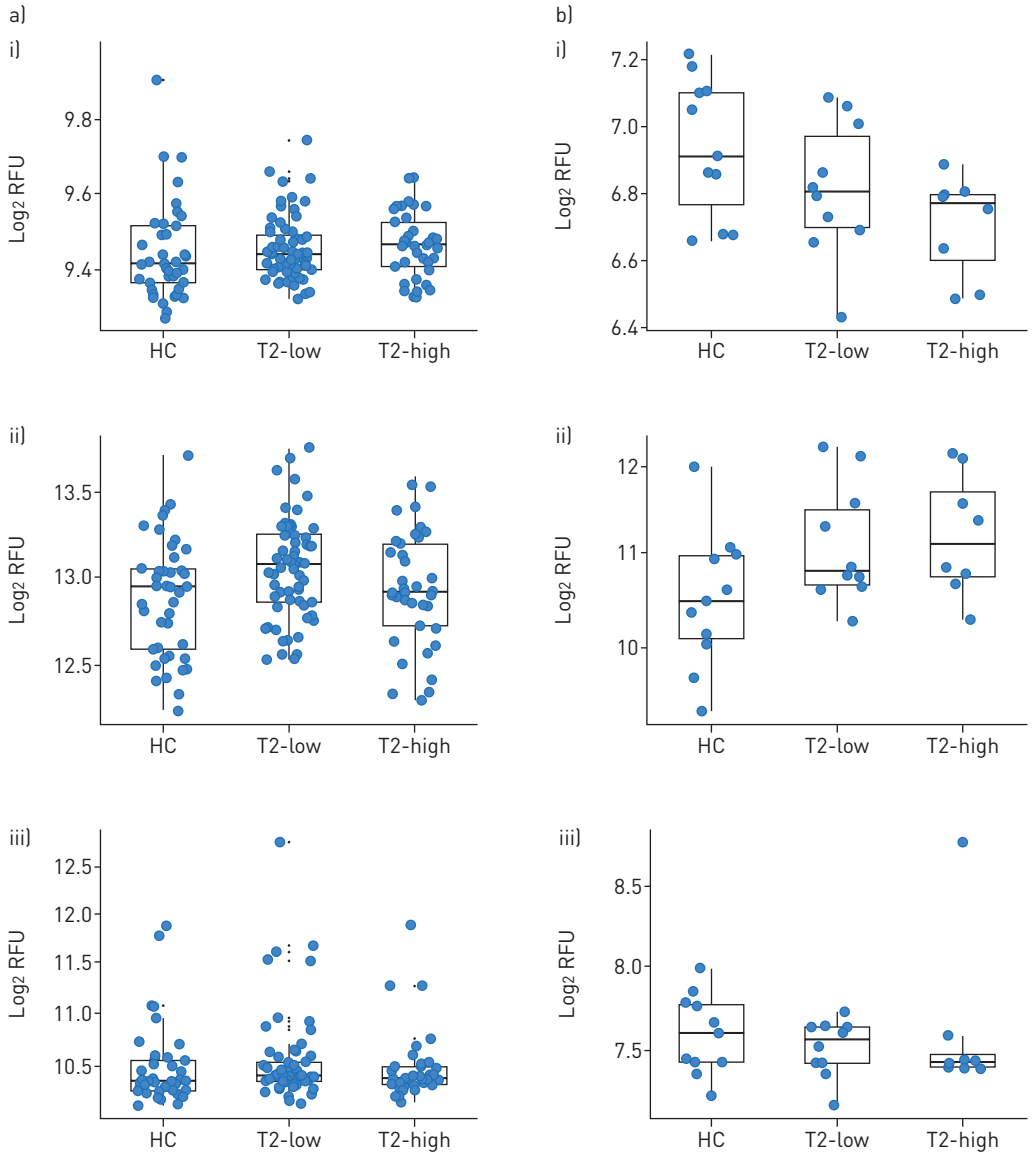

FIGURE 5 Box plot of SOMAscan-measured levels of the T2-cytokines i) interleukin (IL)-4, ii) IL-5 and iii) IL-13, in a) serum and b) sputum supernatants of healthy controls (HC) and T2-low and T2-high asthmatic patients. T2-high and -low groups were defined based on the *in vitro* stimulation of airway epithelial lung cells with (IL)-13 IVS signature. There were no significant differences between any of the groups. RFU: relative fluorescent units.

showed some overlap, in terms of patient selection (supplementary figure S3). CCL26 expression in patients with high *F*eNO, blood eosinophils and periostin were similar (figure 3a). CCL26 expression was highest in patients with high *F*eNO and blood eosinophils (supplementary figure S4). Only *F*eNO-high (⩾30 ppb) patients, but not those with high blood eosinophil counts, showed a significant association with the CCL26 T2-high defined status (supplementary table S3).

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 >95th centile of the expression scores in our nonasthmatic controls, we found that 37% of asthmatic participants (45% nonsmoking severe asthma, 33% of smoking or ex-smoking severe asthma and 28% mild–moderate asthma) were T2-high. Thus, the severe nonsmoking asthma group had the highest expression of T2-high, higher than patients with 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 five-item asthma control questionnaire score. In addition, these patients 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 two-gene *POSTN* and *SERPINB2* expression.

T2-high patients had a higher total serum IgE, *F*eNO, 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–severe asthma group had features indicative of poorer control and more severe asthma, but both T2-high groups shared higher levels of serum IgE, *F*eNO and blood eosinophil counts. However, ROC curve analysis showed that *F*eNO and blood and sputum eosinophil counts were poor predictors of T2-high in severe asthma. The combination of high *F*eNO 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 three genes (*periostin*, *CLCA1* and *SERPINB2*) 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 three 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 was classified as T2-high compared to the application of the IL-13 IVS signature, such that the use of the two-gene signature provided a more conservative result. Only 25.2% of patients could be classified as T2-high using the two-gene signature. Interestingly, in the mild–moderate group, we found a very low percentage of patients to be T2-high (five (14%) out of 36), 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–moderate group, indicating that this signature was more comprehensive.

Other approaches have been previously used to define T2-high asthma. Thus, gene expression of the T2-associated cytokines IL-4, IL-5 and IL-13 has been measured in induced sputum cells using reverse transcriptase-PCR. Using a combined metric of gene expression of these three cytokines, Peters *et al.* [17] 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]. Conversely, we detected measurable levels of the proteins IL-4, IL-5 and IL-13 in sputum supernatants and in serum samples by using the high-throughput SOMAscan protein assay platform which uses 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 CCL18, 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 *F*eNO, 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 noneosinophilic airway inflammation measured in sputum from mild–moderate asthma subjects [21]. The reason for this failure of serum periostin might be related to the fact that periostin produced from the airway epithelium may not effectively reach the systemic circulation. This explanation is supported by unpublished data from the U-BIOPRED cohort, where we have found a better predictability for sputum periostin to distinguish eosinophilic from noneosinophilic asthma. This is the first time that the values of these bedside biomarkers in predicting T2-high status in patients with severe asthma have 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-IL-5 or anti-IL-5R antibody treatments. We examined the use of combined *F*eNO and blood eosinophil count as a predictor of T2-high 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 *F*eNO. 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–severe asthma, it was reported that all subjects with high airway mucosal CC26 gene expression in the epithelium was associated with high *F*eNO 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 *F*eNO 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 one 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.

There are some potential limitations to the study. First of all, we could only perform this analysis in a smaller sample of the whole U-BIOPRED cohort (104 out of 421) who underwent fibre-optic bronchoscopy. This might mean that this sample might not be representative of the whole cohort. However, when we compared the features of those who underwent bronchoscopy *versus* those who did not, we found no significant differences between serum periostin, *F*eNO and blood eosinophil counts, sputum eosinophil counts and serum IgE levels, indicating at least in terms of these biomarkers, this group is representative. Secondly, the U-BIOPRED population may not be representative of asthma in the real world and therefore the findings may not be generalisable. However, it was important to ensure that we used strict criteria to define our patients for this study. Thirdly, we could not obtain definitive answer regarding the influence of active smoking and past smoking on T2 status because of small numbers of asthmatics in these categories, particularly active smokers. We found it difficult to recruit severe asthmatics who were current smokers. Our data suggests that within severe nonsmoking asthma there is an incidence of 45% of T2-high and that in ex-smokers it fell to 25% and in current smokers, it was 50%. However, it is difficult to make any definite comment on the incidence since the numbers of current and ex-smokers were low.

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 IL-13 IVS gene signature. There is a reasonable predictive value of high *F*eNO and high blood eosinophil count in predicting T2-high status, with a combination of high *F*eNO 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. Serum periostin should not be used as a biomarker of T2-high. Better bedside predictor biomarkers for T2-high are therefore needed.

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