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Design and subjects

During the baseline visit data on demographics, medical history, physical examination and lung function were collected to assess inclusion criteria and severity of asthma. Subsequently, questionnaires were completed, atopy status was assessed and blood and sputum collected. Finally, nasal brushings and endobronchial biopsies were collected in a separate visit.

Severe asthma was defined according to the IMI consensus statement [E1], and thereby, patients were included who had a confirmed diagnosis of asthma that was uncontrolled despite high intensity asthma treatment (≥1000µg Fluticasone eq. plus a second controller) or could only be controlled with treatment by systemic oral corticosteroids (OCS) or omalizumab. Uncontrolled asthma was diagnosed if ≥3 of the following features were present: 1) >2 weekly daytime symptoms, 2) any limitations of activities, 3) ≥1 weekly nocturnal symptoms 4) >2 weekly need for reliever medication, 5) pre bronchodilator FEV1 <80% predicted or personal best, or if the patient encountered ≥2 yearly severe exacerbations. Current-smokers, ex-smokers and never-smokers were included.

PAL was diagnosed in patients with a post-bronchodilator FEV1/FVC < lower limit of normal (LLN). The LLN was calculated for each patient according to formulae stated by Quanjer *et al.* [E2] (for male subjects: -0.18*AGE + 75.41; for female subjects: -0.19*AGE + 78.4) consistent to LLN defined by Global Lung Function Initiative (GLI) [E3]. In order to examine consistency with other criteria for PAL we additionally performed an analysis based on PAL defined by post-bronchodilator FEV_1 < LLN. The U-BIOPRED study was registered at ClinicalTrials.gov identifier NCT01976767 and was approved by the Medical Ethics Boards of all participating centers. All patients provided written informed consent.

Measurements

Clinical data

Clinical data were collected according to predefined standard operating procedures (SOPs) and details on the methods of collection in U-BIOPRED have been published elsewhere [E4]. First, by history taking data on age, gender, body mass index (BMI), age of onset of asthma, medication and smoking history were assessed. Second, asthma symptoms were obtained by a questionnaire (ACQ)[E5]. Third, lung function was measured pre- and post-bronchodilator and according to standardized procedures [E6]. Fourth, eosinophil and neutrophil counts were assessed in blood and induced sputum samples.

mRNA samples

Gene expression was assessed on the total RNA in unselected samples of severe asthma patients in the following four different airway compartments: (1) nasal brushing (n=37), (2) induced sputum (n=79), (3) endobronchial brushings (n=62) and (4) endobronchial biopsies (n=50). There was no complete overlap between the samples, mainly because of limited sputum induction success rate (Fig. 1).

Sputum induction

Sputum samples, induced by inhalation of hypertonic saline, were processed with 0.1%DTT by using the selected sample technique, quality controlled according to ERS recommendations [E7]. Sputum was processed within 2 hours after collection and processed suspension was preserved in RNAlater® solution and then maintained at -80°C.

Nasal brushing, endobronchial brushing and biopsy

Nasal brushings were collected from one nostril (4mm plastic coated wire interdental brush (DENT.O.CARE Limited, 7 Cygnus Business Centre, Dalmeyer Road, London, UK)). Samples were embedded in phosphate buffer saline (PBS) directly after the procedure. Bronchoscopy procedures were based on U-BIOPRED SOPs and performed according to safety standards [E8]. Patients had refrained smoking ≥6 hours prior to the procedure, and subsequently received bronchodilator medication and local anesthesia. A flexible scope (type of scope depending on preferences of physician and clinical center) was introduced and, first, four endobronchial brushings were performed in a large airway (bronchus intermedius) contacting the wall at least 4 times (with e.g. Olympus REF: BC-202D-2010 (2mm brush size) or BC-202D-3010 (3mm brush size), KeyMed (Medical & Industrial Equipment, Ltd OLYMPUS Group Company)). Subsequently, up to 8 endobronchial biopsies were taken from the 2nd and 4th airway carinae of the right or left lower and middle lobes, working upwards (with a disposable 1.8 mm cupped biopsy forceps). Nasal brushings and endobronchial biopsies and brushings were immediately preserved in RNAlater® solution and then maintained at -80°C. RNA was extracted using Qiagen miRNeasy kit (Qiagen; Germantown, MD) and amplified with NuGen ovation pico WTA kit (NuGen Technologies; San Carlos, CA).

mRNA microarray analysis

Microarray analysis was performed with the Affymetrix HT HG-U133+ PM microarray platform (Affymetrix, Santa Clara, Ca). Pre-processing and quality control were performed with multi-array average normalization (Almac, Craiganvond, UK) and the obtained CEL files were normalized. Technical outliers were excluded (chip image analysis, Affymetrix GeneChip QC, RNA degradation analysis, distribution analysis, principal components analysis, and correlation analysis) and CEL files re-normalized using the robust multi-array (RMA) method. Technical batch effects (e.g., from microarray hybridization date/lot, RNA processing batch) were adjusted in the data matrices using linear modeling of batch (as random factor).

Statistical analysis

Clinical variables were summarized as mean±standard deviation when normally distributed, as median (interquartile range) when skewed and as their frequencies (proportion) when categorical. Between group comparison was performed with independent t-tests, Mann-Whitney U test or chi-square tests, as appropriate. Clinical variables with a $p<0.05$ were considered significantly different.

GSVA

Gene set variation analysis (GSVA) is a statistical technique that allows sensitive identification of differences in expression of sets of genes between heterogeneous groups and can be used to explore for underlying pathways [E9]. Sets of genes were predefined, and were based on the available gene expression publications and data on airways disease, including human and murine models, both *in vivo* and *in vitro* studies, to assure sensitive pathway detection. These included studies on the gene expression associated with (1) airway disease treatment, (2) immunologic pathways and (3) induced lung injury or inflammation. The latter included gene signatures that were identified at several time points after the admission of either Poly(I:C) as a model for exacerbations [E10], or bleomycin, which is used as a model for mimicking the course of fibrotic processes in the lung [E11]. For this discovery study 105 predefined gene sets were entered into the statistical models (Table E7).

Enrichment scores and false discovery

Enrichment scores (ES) were calculated for each patient and for each of the gene signatures and were based on the gene expression of genes in the sets. ES could range from a value of -1 to 1 [E9]. Subsequently, mean ES were calculated for patients with PAL and patients without PAL and Generalized Linear Models, including correction for smoking status, corticosteroid usage and duration of asthma, were applied to statistically compare ES between the groups. In order to minimize false discovery, only gene signatures that had $p<0.05$ and a difference of ES (dES) between

of the groups of ≥0.2 were considered significantly different, following the Microarray Consortium for Quality Control (MACQC) recommendations regarding the need for applying group-difference thresholds in order to stringently limit false discovery [E12].

Consistency when using different definition of PAL

211 out of 421 patients (50.1%) were having PAL defined as FEV_1 < LLN. Patient characteristics were similar in both analyses, however patients with PAL defined as FEV_1 < LLN were also more frequent female and had a higher ACQ score (Table E5).

GSVA analysis showed that identified signatures between FEV_1/FVC < LLN and FEV_1 <LLN were also similar, including those associated with treatment with fluticasone, eosinophilic inflammation and involvement of T_H2 helper cells and IFN-alpha. In addition, induced lung injury and inflammation gene signatures were identified in both analyses as well (Table E6). However, involvement of CD4 T-cells of rheumatoid arthritis was not consistent, and no significant gene signatures were identified in the biopsies applying FEV_1 < LLN.

107

Table E1: Patient Characteristics of Nasal Brushings sample

	No PAL	PAL	*P*-Value
n	15	22	
Gender = Female (%)	**11 (73.3%)**	**8 (36.4%)**	**0.045**
Age (yrs)*	47.5 ± 16.2	53.2 ± 12.1	0.229
BMI (Kg/m^2)*	34.1 ± 6.6	29.4 ± 5.7	0.027
Asthma Duration (yrs)*	25.0 [14.0 - 38.0]	21.5 [17.0 - 40.5]	0.914
Smoking Status (%)			0.188
Never Smokers	12 (80.0)	11 (50.0)	
Ex-smokers	3 (20.0)	8 (36.4)	
Current-smokers	0 (0.0)	3 (13.6)	
Packyears†	5.0 [3.3 - 5.1]	20.0 [11.5 - 38.5]	0.052
OCS dose (mg)†	10.0 [8.0 - 25.0]	10.00 [8.12, 13.75]	0.616
ACQ*	1.9 ± 1.0	2.2 ± 1.2	0.414
Exacerbations per year†	2.0 [1.0 – 4.0]	3.0 [1.0 - 4.0]	0.982
pbFEV$_1$ (% predicted)*	**99.4 ± 17.6**	**71.5 ± 14.9**	**<0.001**
Blood Eosinophils (x10^3/L)†	0.2 [0.1 - 0.4]	0.2 [0.1 - 0.3]	0.817
Blood Neutrophils (x10^3/L)†	**3.8 [3.6 - 5.1]**	**5.0 [4.2 - 7.8]**	**0.033**
Sputum Eosinophils (%)†	1.4 [0.4 - 4.6]	2.6 [0.2 - 13.8]	0.614
Sputum Neutrophils (%)*	46.6 ± 18.4	63.2 ± 18.8	0.053

* mean ± SD; † median [Interquartile Range]; PAL: persistent airflow limitation; OCS: Oral corticosteroids; ACQ: Asthma Control Questionnaire FEV$_1$: Forced Expiratory Volume in the first second

108

109

110

Table E2: Patient Characteristics of sputum sample

	No PAL	PAL	*P*-Value
n	21	58	
Gender = Female (%)	13 (61.9%)	35 (60.3%)	1.000
Age (yrs)*	52.5 ± 13.9	54.2 ± 11.4	0.580
BMI (Kg/m^2)*	28.2 ± 5.0	28.1 ± 5.3	0.928
Asthma Duration (yrs)*	23.1 ± 14.5	26.8 ± 17.2	0.374
Smoking Status (%)			0.799
Never Smokers	11 (52.4%)	35 (60.3%)	
Ex-smokers	8 (38.1%)	18 (31.0%)	
Current-smokers	2 (9.5%)	5 (8.6%)	
Packyears†	13.8 [2.9 - 19.7]	10.0 [2.8 - 18.8]	0.922
OCS dose (mg)†	10.0 [8.8 - 12.5]	10.0 [6.9 - 13.1]	0.691
ACQ*	2.6 ± 1.4	2.6 ± 1.3	0.914
Exacerbations per year†	2.0 [2.0 – 3.5]	2.0 [1.5 – 4.0]	0.833
pbFEV$_1$ (% predicted)*	**90.2 ± 14.0**	**63.5 ± 19.9**	**<0.001**
Blood Eosinophils (x10^3/L)†	0.2 [0.2 - 0.3]	0.4 [0.2 - 0.5]	0.203
Blood Neutrophils (x10^3/L)†	4.3 [3.3 - 6.3]	5.0 [3.9 - 7.5]	0.051
Sputum Eosinophils (%)†	**1.3 [0.2 - 3.8]**	**6.2 [1.3 - 28.5]**	**<0.001**
Sputum Neutrophils (%)*	61.5 ± 23.6	58.5 ± 27.1	0.663

* mean ± SD; † median [Interquartile Range]; PAL: persistent airflow limitation; OCS: Oral corticosteroids; ACQ: Asthma Control Questionnaire FEV$_1$: Forced Expiratory Volume in the first second

111

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Table E3: Patient Characteristics of endobronchial brushings sample

	No PAL	PAL	*P*-Value
n	30	32	
Gender = Female (%)	**19 (63.3)**	**10 (31.2)**	**0.021**
Age (yrs)*	46.7 ± 14.0	53.0 ± 11.1	0.052
BMI (Kg/m^2)*	31.3 ± 6.3	28.5 ± 6.1	0.081
Asthma Duration (yrs)*	26.4 ± 16.8	26.7 ± 17.9	0.940
Smoking Status (%)			0.077
Never Smokers	22 (73.3)	15 (46.9)	
Ex-smokers	7 (23.3)	12 (37.5)	
Current-smokers	1 (3.3)	5 (15.6)	
Packyears†	**5.1 [1.4 - 8.1]**	**18.8 [7.0 - 24.0]**	**0.031**
OCS dose (mg)†	10.0 [10.0 - 21.3]	10.0 [6.5 - 16.3]	0.246
ACQ*	2.1 ± 1.2	2.4 ± 1.0	0.314
Exacerbations per year†	2.0 [2.0 – 4.0]	3.0 [2.0 – 4.0]	0.768
pbFEV$_1$ (% predicted)*	**91.9 ± 14.3**	**66.7 ± 15.5**	**<0.001**
Blood Eosinophils (x10^3/L)†	0.2 [0.1 - 0.4]	0.2 [0.1 - 0.3]	0.319
Blood Neutrophils (x10^3/L)†	**4.0 [3.2 - 5.4]**	**5.8 [4.1 - 7.7]**	**0.005**
Sputum Eosinophils (%)†	1.4 [0.6 - 4.6]	4.5 [0.2 - 24.9]	0.520
Sputum Neutrophils (%)*	48.5 ± 20.7	61.6 ± 23.3	0.143

* mean ± SD; † median [Interquartile Range]; PAL: persistent airflow limitation; OCS: Oral corticosteroids; ACQ: Asthma Control Questionnaire FEV$_1$: Forced Expiratory Volume in the first second

114

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Table E4: Patient Characteristics of endobronchial biopsies sample

	No PAL	PAL	*P*-Value
n	22	28	
Gender = Female (%)	14 (63.6%)	12 (42.9%)	0.166
Age (yrs)*	48.4 ± 12.6	52.7 ± 11.2	0.212
BMI (Kg/m^2)*	30.2 ± 5.4	28.6 ± 6.4	0.344
Asthma Duration (yrs)*	25.8 ± 15.5	30.5 ± 18.7	0.353
Smoking Status (%)			0.245
Never Smokers	16 (72.7%)	16 (57.1%)	
Ex-smokers	5 (22.7%)	6 (21.4%)	
Current-smokers	1 (4.5%)	6 (21.4%)	
Packyears†	**5.3 [2.0 - 13.4]**	**21.5 [18.0 - 27.5]**	**0.049**
OCS dose (mg)†	10.0 [7.9 - 10.0]	10.0 [6.0 - 15.0]	0.966
ACQ*	2.3 (1.3)	2.4 (1.1)	0.721
Exacerbations per year†	2.0 [2.0 – 3.3]	3.0 [2.0 – 4.0]	0.249
pbFEV$_1$ (% predicted)*	**89.6 ± 13.6**	**68.3 ± 15.2**	**<0.001**
Blood Eosinophils (x10^3/L)†	0.2 [0.1 - 0.3]	0.2 [0.1 - 0.3]	0.683
Blood Neutrophils (x10^3/L)†	4.0 [3.5 - 5.3]	5.0 [4.3 - 7.3]	0.050
Sputum Eosinophils (%)†	2.0 [0.7 - 8.0]	2.6 [0.2 - 6.1]	0.938
Sputum Neutrophils (%)*	50.4 ± 19.5	56.5 ± 27.0	0.518

* mean ± SD; † median [Interquartile Range]; PAL: persistent airflow limitation; OCS: Oral corticosteroids; ACQ: Asthma Control Questionnaire FEV$_1$: Forced Expiratory Volume in the first second

118

119

120

Table E5: Patient characteristics of patients with persistent airflow limitation (PAL) defined as post-bronchodilator FEV1 < LLN

	no PAL	PAL	*P*-value
n	**210**	**211**	
Female (%)	**118 (56.2)**	**143 (67.8)**	**0.016**
Age (yrs)*	**49.7 ± 14.8**	**54.1 ± 11.6**	**0.001**
BMI (Kg/m2)*	29.3 ± 6.3	29.2 ± 6.3	0.825
Asthma Duration (yrs)†	**20.0 [11.0 - 30.0]**	**26.0 [13.3 - 41.8]**	**<0.001**
Smoking history (%)			0.832
Never	133 (63.3)	131 (62.1)	
Ex-smoker	58 (27.6)	57 (27.0)	
Current smoker	19 (9.0)	23 (10.9)	
Packyears†	12.5 [4.5 - 20.0]	12.7 [3.8 - 21.4]	0.975
OCS dose†	12.8 ± 8.6	14.3 ± 10.6	0.345
ACQ*	**2.3 ± 1.3**	**3.0 ± 1.2**	**<0.001**
Exacerbations per year †	2.0 [1.0 - 4.0]	2.0 [2.0 - 3.0]	0.568
pbFEV1 (% pred)*	**91.5 ± 13.6**	**60.2 ± 15.1**	**<0.001**
Blood Eosinophils (x109/L)†	0.2 [0.1 - 0.4]	0.2 [0.1 - 0.4]	0.950
Blood Neutrophils (x109/L)†	4.9 [3.8 - 6.4]	4.8 [3.6 - 7.3]	0.969
Sputum Eosinophils (%)†	2.0 [0.4 - 9.6]	3.8 [1.0 - 20.7]	0.099
Sputum Neutrophils (%)*	51.7 [31.5 - 72.8]	56.5 [37.4 - 74.4]	0.298

* mean ± SD; † median [Interquartile Range]; PAL: persistent airflow limitation; OCS: Oral corticosteroids; ACQ: Asthma Control Questionnaire; FEV_1: Forced Expiratory Volume in the first second

121

Table E6: Differentially enriched gene signatures in severe asthma patients with FEV1 post-bronchodilator < LLN as compared to severe asthma patients without FEV1 post-bronchodilator < LLN

	Gene signatures associated with	Nasal brush		Sputum		Endobr. Brush		Endobr. Biopsy	
		dES	p-value	dES	p-value	dES	p-value	dES	p-value
Treatment gene signatures	Fluticasone treatment in asthma - DOWN[1]*					0.23	0.007		
	Fluticasone treatment in asthma - UP[1]*					0.23	0.009		
	Asthma (HDM induced model) - UP[3]					0.21	0.009		
Immunologic gene signatures	Eosinophils - UP[1]*			0.37	0.001	0.21	0.039		
	TH2 activated - DOWN[2]*	-0.21	0.033						
	IFN-alpha - UP[1]*			*-0.19*	0.011				
Induced lung injury gene signatures	Induced inflammation (Poly I:C - 72h) - UP[3]*			-0.21	0.002				
	Induced injury (Bleomycin - Day 2) - UP[3]*			*-0.19*	0.003				

Differences in mean gene signature enrichment scores between severe asthma patients with FEV1 post-bronchodilator < LLN as compared to severe asthma patients without FEV1 post-bronchodilator < LLN (in RED are higher and in BLUE are lower). * Comparable results as found in PAL defined as Fev1/FVC < LLN; [1]In vitro model in human sample; [2]In vivo model in human sample; [3]In vivo model in murine model

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178

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180