

# **Differential distribution of inflammatory cells in large and small airways in smokers**

Salvatore Battaglia, Thais Mauad, Annemarie M. van Schadewijk, Antonio M. Vignola, Klaus F. Rabe, Vincenzo Bellia, Peter J. Sterk and Pieter S. Hiemstra

On-line Data Supplement

## **Methods (additional online details)**

### **Subjects and Study Design**

Consecutive patients were selected from those scheduled in the Department of Thoracic Surgery of the LUMC and prospectively enrolled in this cross-sectional study. Patients were smokers or ex-smokers scheduled for surgical resection for primary lung cancer. Those subjects who underwent surgery for other diseases were excluded. Fifteen smokers were recruited (table 1), including seven current smokers and eight ex-smokers, who had stopped smoking at least six months before the lung resection.

None of the patients reported exacerbations of their disease during the 2 months preceding the study. Four patients had received inhaled glucocorticoids and one of these patients had also received oral steroids in the days preceding the surgical resection.

The study was approved by the Medical Ethics Committee of the LUMC and informed written consent was obtained for each subject.

### **Histology**

#### **Tissue Sampling and Processing**

At least two samples of peripheral parenchyma and one or two samples of central airways free of tumour were obtained. Tissue samples were fixed in 4% formaldehyde for 24 hours. Paraffin embedded tissue was cut in 4- $\mu$ m thick sections. Histological exclusion criteria were: microscopic infiltration by tumour, obstructive pneumonia or fibrotic diseases.

One or two large airways (LA) and all transversally cut small airways (SA), defined by internal perimeter larger (LA) or smaller (SA) than 6 mm, were selected for each patient [6][13]. To avoid measurements in tangentially-cut airways, airways with a short/long diameters ratio less than 0.33 were excluded from the study.[14]

## **Inflammatory cells**

Mouse monoclonal antibodies were used for identification of neutrophils (anti-human neutrophil elastase, clone NP57, Dako, Glostrup, Denmark), macrophages (clone KP1, anti-CD68, Dako), CD4<sup>+</sup> cells (clone 4B12, anti-CD4, Novocastra; Newcastle upon Tyne, UK), CD8<sup>+</sup> cells (clone 1A5, anti-CD8, Novocastra), and mast cells (tryptase, AA1, Dako). A polyclonal antibody was used for the identification of total T lymphocytes (rabbit-anti-CD3, Dako). Citrate buffer was used for antigen retrieval for all antibodies, except for neutrophil elastase. Peroxidase labeled mouse- or rabbit Envision (Dako) was used as a secondary antibody, and Nova Red (Vector laboratories; Burlingame, CA) was used as a chromogen. In the negative controls, the primary antibody was omitted from the procedure and PBS was used instead.

## **Morphometric analyses**

Cases were coded and the measurements were done without knowledge of the clinical data. For each airway as many images as needed to cover the whole perimeter were acquired at 200X magnification. Lamina propria (or inner-wall) was defined as the zone between the epithelial basement membrane and the smooth muscle. The cellular infiltrate was quantified in the lamina propria of SA and LA using a fully automated image analysis system (Kontron/Zeiss Vision KS-400 system; Carl Zeiss, Göttingen, Germany).[15] The number of positively stained cells was counted using an automated method, which has been shown to be fully reproducible and to have good agreement with numbers obtained using interactive cell counting.[15] Data were expressed as numbers of cells per mm<sup>2</sup>.

## Additional on-line Result

In this on-line supplement the results of subgroup analyses are presented in tables. For this purpose, we compared subjects that are current smokers (CS, n=7) and ex-smokers (ES, n=8). In this latter group only subjects were included who had stopped smoking at least six months before the lung resection surgery. Furthermore, results were compared from subgroups of patients with fixed airway obstruction (COPD, n=9) and without COPD (Non-COPD, n=6) that were identified on the basis of airflow limitation defined as of forced expiratory volume in one second (FEV<sub>1</sub>) <80% of predicted. In table E1 the patient characteristics are given according to subgroup division, all COPD patients were in GOLD stage II (moderate) with FEV1 ranging between 54% to 80% of predicted.

Results reported in this section should be interpreted with caution due to the small number of patients in each subgroup.

Table E2 shows differences in neutrophils and mast cells between small (SA) and large airways (LA). The density of these cells is higher in SA compare to LA, both in the whole sample (see main text: results and figure 1) as well as in all subgroups (except for mast cells in non-COPD subgroup).

Densities of lymphocytes and macrophages in the lamina propria were not statistically different between SA and LA in all subgroups (table E3). As reported in the main text (see results and figure 1) the CD4<sup>+</sup> cell content was higher in the lamina propria of LA compared to SA.

We next explored whether inflammatory cell infiltration in large and small airways is associated with smoking status or with the development of the airflow limitation. Interestingly, when comparing current smokers to ex-smokers, a higher number of CD8<sup>+</sup> cells was found in the SA of current smokers (median: 330.5; range: 128.9-784.8

cells/mm<sup>2</sup>) compared to ex-smokers (median: 175.1; range: 0.0-282.9 cells/mm<sup>2</sup>; p=0.009) (table E3). While the mean numbers of CD4+ cells in small airways of ex-smokers was higher than in the current smokers, this difference did not reach statistical significance (p=0.054). Consequently, a lower CD4<sup>+</sup>/CD8<sup>+</sup> ratio was found in the SA of current smokers (median: 0.24; range: 0.02-0.41) compared to the SA of the ex smokers (median: 1.42; range: 0.26-2.73; p=0.002) (table E4).

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No differences for lymphocytes were found in the large airways, nor for the other cell types comparing smokers to ex-smokers, or COPD to non-COPD patients in both large and small airways. Interestingly the neutrophil numbers in small airways of COPD patients were markedly higher when compared to those of non-COPD, however this difference did not reach statistical significance (p=0.066).

## Brief on-line discussion

In our analysis, we have also included a subgroups analysis of smokers with COPD vs smokers without COPD and in ex-smokers vs smokers. Although the results need to be interpreted with cautions because the subgroups were small in size, nevertheless the same pattern of distribution was observed (increased neutrophils and mast cell density in LA vs SA airways). These data indicate that the inflammatory cell distribution within the airways may not be affected by the development of obstruction or smoking cessation. Interestingly, there were no significant differences in cell density between the groups which may be explained by the small sample size.

Table E1: Patient characteristics.

	All	Ex-smokers	Current smokers	COPD	Non-COPD
Number	15	8	7	9	6
Age*	61.1±10.5	66.6±8.1\$	54.7±9.7	63.0±8.9	58.2±12.9
Sex (F/M)	3/12	1/7	2/5	1/8	2/4
FEV <sub>1</sub> %Pred*	79.3±15.3	77.0±13.6	82.0±17.7	69.0±9.4 ‡	94.8±5.7
FEV <sub>1</sub> /FVC*	70.6±9.3	71.5±8.2	69.6±11.1	66.5±9.9 †	76.8±3.3
Pack yrs*	40.0±22.9	36.3±13.3	45.0±32.6	33.5±14.2	48.7±30.3
Steroid inh. (yes/no)	4/11	1/7	3/4	4/5	0/6
Steroid or. (yes/no)	1/14	0/8	1/6	1/8	0/6

\* Data as mean ± SD

\$ = p=0.022 compared to current-smokers

† = p=0.031 compared to non-COPD

‡ = p< 0.0001 compared to non-COPD

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Table E2: Neutrophils and mast cells in the lamina propria (cells/mm<sup>2</sup>) of large and small airways.

		Neutrophils		Mast Cells	
Group	#	LA	SA	LA	SA
<b>All</b>	15	60.2 (9.0-743.3)	225.3 ‡ (42.1-874.9)	133.7 (43.0-292.0)	313.3 ‡ (112.9-507.5)
<b>ES</b>	8	113.8 (15.7-743.3)	298.5 \$ (169.9-874.9)	145.3 (43.0-262.8)	300.6 \$ (155.9-469.8)
<b>CS</b>	7	39.0 (9.0-195.0)	190.0 \$ (42.1-644.7)	109.7 (79.5-292.0)	313.3 \$ (112.9-507.5)
<b>COPD</b>	9	58.8 (15.7-352.8)	304.9 † * (172.7-813.6)	133.7 (97.9-262.8)	335.7 † (155.9-419.0)
<b>Non-COPD</b>	6	86.2 (9.0-743.3)	176.4 \$ (42.1-874.9)	134.5 (43.0-292.0)	255.2 (112.9-507.5)

Data are expressed as median (range). Abbreviations: LA = large airways; SA = small airways; ES = ex-smokers; CS = current smokers.

\$ = p< 0.05 compared to the same cell type in LA.

† = p< 0.01 compared to the same cell type in LA.

‡ = p< 0.001 compared to the same cell type in LA.

\* = p=0.066 compared to non-COPD

Table E3: Lymphocytes and macrophages in the lamina propria: cells/mm<sup>2</sup>

Group	#	CD3+		CD4+		CD8+			
		LA	SA	LA	SA	LA	SA		
<b>All</b>	15	442.0 (167.1-932.8)	497.3 (61.5-1441.9)	217.8 * (0.0-903.4)	80.5 (6.6-452.9)	236.1 (99.8-895.5)	237.1 (0.0-784.8)	(	
<b>ES</b>	8	437.6 (200.6-888.1)	545.7 (61.5-1441.9)	373.8 (164.4-903.4)	273.5 (48.6-452.9)	243.4 (99.8-582.3)	175.1 (0.0-282.9)	(	
<b>CS</b>	7	446.4 (167.1-932.8)	333.1 (141.4-861.3)	199.9 (0.0-561.5)	68.6 (6.6-152.3)	207.5 (108.8-895.5)	330.5 † (128.9-784.8)	(	
<b>COPD</b>	9	442.0 (167.1-932.8)	517.9 (141.4-1441.9)	304.8 (26.2-598.1)	102.3 (53.2-452.9)	236.1 (108.8-500.7)	212.3 (103.1-330.5)	(	
<b>Non COPD</b>	6	540.6 (200.6-873.4)	429.7 (61.5-861.3)	190.0 (0.0-903.4)	65.1 (6.6-321.3)	384.6 (99.8-895.5)	324.8 (0.0-784.8)	(	

Data are expressed as median (range). Abbreviations: LA = large airways; SA = small airways; ES = ex-smokers; CS = cu

\* = p < 0.042 compared to the same cell type in SA.

† = p = 0.009 compared to ex-smokers.

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Table E4: CD4<sup>+</sup>/CD8<sup>+</sup> cell ratio in the lamina propria: cells/mm<sup>2</sup>

Group	#	CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	
		LA	SA
All	15	0.94 (0.0-3.89)	0.36 (0.02-2.73)
ES	8	1.59 (0.68-2.17)	1.42 (0.26-2.73)
CS	7	0.66 (0.0-3.89)	0.24 † (0.02-0.41)
COPD	9	0.94 (0.24-3.89)	0.41 (0.24-2.73)
Non COPD	6	1.23 (0.0-1.81)	0.19 (0.02-1.37)

Data are expressed as median (range).

Abbreviations: LA = large airways; SA = small airways; ES = ex-smokers; CS = current smokers.

† = p=0.002 compared to ex-smokers.

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