Clinical control of asthma is associated with measures of airway inflammation

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Supplements for Web-only publication

Supplements for Web-only publication: METHODS

Collection, processing and immunohistochemical staining of bronchial biopsies

The inflammatory profile of 11 asthmatic subjects was assessed with specific antibodies against eosinophilic peroxidase (EPX, laboratories of NA Lee and JJ Lee, Mayo Clinic, Scottsdale, Arizona, USA), mast cell tryptase (AA1, DAKO, Glostrup, Denmark), macrophages (CD68, DAKO), neutrophil elastase (NP57, DAKO) and T-lymphocytes (CD3 (DAKO), CD4 (Novocastra, Newcastle upon Tyne, UK) and CD8 (DAKO). In short, sections were deparaffinized, antigens were retrieved, and the sections then incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) followed by the chromogen NovaRED (Vector Labs, Burlingame, California, USA). EPX and CD8 were detected using biotinylated anti-mouse IgG1 (Southern Biotechnology, Birmingham, Alabama, USA) and alkaline phosphatase- (DAKO) or peroxidase-labeled streptavidine conjugates (DAKO), followed by permanent Red (DAKO) and NovaRed chromogens, respectively. Epithelial proliferation was assessed by Ki67 expression and submucosal deposition of extracellular matrix (ECM) by collagen 3 expression. In short, sections were deparaffinized, antigens were retrieved, and the sections incubated with antibodies against Ki67 (DAKO) and collagen 3 (Southern Biotechnology). These antibodies were detected with two peroxidase-labeled conjugates (both DAKO). 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used since chromogen and hematoxylin (Ki67) or methylgreen (collagen 3) were used for counterstaining. All stainings (except for Ki67) were performed in an automated system using the DAKO autostainer in three consecutive runs per cell marker. Sections were manually counterstained with methylgreen.

Quantification

Quantification of inflammatory cells was performed by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). The largest of three biopsy samples was generally chosen for quantification. The number of positively stained inflammatory cells was counted in the submucosal area 100 µm under the basement membrane (BM), in a total area of 0.1 mm^2 per biopsy sample. Additionally, activation of eosinophils and mast cells was determined by their degree of degranulation. EPX staining showed widely spread distribution of eosinophilic granules, not necessarily in close proximity to EPX^+ cells. Therefore, degranulation of eosinophils was determined by quantification of the EPX immunopositive area by computer-assisted image analysis. $AA1^+$ mast cell granules were only observed in close proximity to $AA1^+$ cells and, therefore, the percentage of degranulated $AA1^+$ cells was determined by counting (Carroll, N.G. et al. *Eur. Respir. J.*, 2002. 19:879-885). In short, positively stained nucleated mast cells were classified as intact if they were dense, compact, had unbroken boundaries, and did not have any surrounding positively stained granules. All other nucleated $AA1^+$ cells were classified as degranulated.

BM thickness was calculated by dividing the BM surface area by the BM length. Epithelial integrity was determined using hematoxylin and eosin-stained biopsy sections and expressed as a percentage of the BM covered with: (1) normal epithelium (a layer of basal and ciliated columnar epithelial cells without detachment from the BM), (2) basal epithelial cells only, (3) no epithelium: denuded BM, or (4) metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells).

The number of goblet cells was counted on PAS-stained biopsy sections and expressed per mm of BM. Additionally, the number of PAS-positive pixels was determined in the

epithelium and expressed as the percentage of mucus-positive epithelium per biopsy section. Epithelial thickness was determined by dividing the epithelial surface area by the BM length. Epithelial proliferation was determined by counting the number of Ki67-positive epithelial nuclei in intact and basal epithelium. Expression of collagen 3 was measured by computer-assisted image analysis in the submucosal area 200 μm under the BM, and was expressed as the percentage of positive tissue per biopsy section.

Online data supplement: **RESULTS**

Multiple logistic regression on Asthma Control

Multivariate logistic regression analysis was used to reveal which variables determine the level of control of asthma. However, due to multi-co-linearity, not all the variables related to eosinophilic inflammation (alveolar NO, slope AMP, eosinophils in sputum and EPX pixels in biopsies) could be entered simultaneously into the model. We therefore created an alternative model including sex, age, Phadiatop ratio, inhaled corticosteroid (ICS) use and FEV_1/VC. We entered the following variables separately into this model: alveolar NO, blood eosinophils, slope AMP, EPX-positive pixels or the percentage intact epithelium. In most models (4 out of 5) Phadiatop ratio and ICS use were found to be significant determinants of asthma control. The two exceptions were when alveolar NO was entered, ICS use was no longer a significant determinant, and when slope AMP was entered, Phadiatop ratio was no longer a significant determinant for asthma control. Importantly, the AMP slope (odds ratio (OR) 0.39; 95% confidence interval (CI) 0.24-0.63) and EPX-positive pixels (OR 0.32; 95%CI: 0.13-0.79) were also found to be significant determinants for asthma control.

TABLE E1. FAILURE OF ASTHMA CONTROL DURING 4 WEEKS FOLLOW-UP

	Criteria of asthma control		No of patients that failed the criteria[Ψ]			Median (range) values per group		
	Totally Controlled	Well Controlled	Total (n=22)	Well (n=47)	No (n=42)	Total	Well	No
During 4 weeks	**All of:**	**Two or more of:**						
Daytime symptoms n (%)[¶]	None	≤ 2 days with score >1	0	26 (55)	12 (29)	0[^^^]	0.18 (0-1.4)	1.0 (0-4.0)*** [†††###]
Rescue beta-2 agonist use n (%)[¶]	None	≤ 2 days & ≤ 4 occasions	0	9 (19)	20 (48)	0[^^]	0 (0-2)	0.4 (0-3.3) *** [†††###]
Morning PEF (%)[¶]	≥ 80% pred. every day	≥ 80% pred. every day	0	24 (51)	32 (76)	97 (88-124)[^^]	87 (41-128)	81 (40-99)***[###]
	AND each week all of the following							
Night-time awakening[‡]	None	None	0	0	21 (50)	0	0	2 (0-41) *** [†††###]
Exacerbations[‡]	None	None	0	0	0	0	0	0
Emergency visits[‡]	None	None	0	0	0	0	0	0
Treatment-related AEs[‡]	None enforcing change in asthma therapy	None enforcing change in asthma therapy	0	0	0	0	0	0

[Ψ]Numbers of occasions that patients failed to fulfil the criteria of totally controlled or well controlled. Values in the well controlled asthma group are the number of occasions that patients do not fulfil the criteria of total control. Values in the uncontrolled asthma group are the number of occasions that patients do not fulfil the criteria of being well controlled. [¶] average per day; [‡] number of events during 4 weeks. Daytime symptom score: symptoms for 1 short period during the day (overall scale: 0 [none] to 5 [severe]). Exacerbations: deterioration in asthma requiring treatment with oral corticosteroid or visit to emergency department, hospitalization, or both. AE adverse event; *** p<0.01 versus controlled; [†††] p<0.001 versus well-control; [##] p<0.01 or [###] p<0.001 versus totally control; [^^] p<0.01 or [^^^] p<0.001 versus well controlled.

Table E2. Inflammation in sputum

	Controlled Asthma		Uncontrolled Asthma
	Totally controlled (n=22)	Well controlled (n=47)	Uncontrolled (n=42)
*Sputum **evaluable***	13 (59%)	34 (72%)	33 (79%)
Total cells (x10^6/mL)	0.47 (0.07-1.79)	0.54 (0.05-3.0)	0.37 (0.14 – 3.86)
Neutrophils (%)	60.9 (21.6-84.9)	59.7 (15.6-94.0)	55.8 (16.8-88.8)
Macrophages (%)	34.1 (13.1-76.5)	36.3 (4.9-82.5)	35.1 (9.1-66.7)
Lymphocytes (%)	0.2 (0 – 2.4)	0.5 (0-2.4)	0.3 (0.0–5.7)
Eosinophils (%)	1.4 (0- 5.7)	0.8 (0-38.6)	1.2 (0-67.1)
Eosinophil score (%)	80 (40-100)	80 (20-100)	80 (20-100)
Subjects with			
% eosinophils >1.9%	n=3 (23%)	n=6 (18%)	n=13 (42%)*
% eosinophils >3.0%	n=3 (23%)	n=4 (12%)	n=12 (36%)*

Values are medians (ranges). No significant differences between controlled versus uncontrolled groups, or between totally controlled versus well controlled. Absolute numbers of inflammatory cells in sputum were also similar between the three groups. Eosinophil score based on Boulet[1] (% eo's categorized in four groups 0%=100%, <=2%=80%, 2-5%=60%, 5.1-8%=40%, >8%=20%). *: $p<0.05$ vs controlled asthma (Chi-square).

Reference 1: Boulet L, Boulet V, Milot J. How should we quantify asthma control? A proposal. Chest 2002;122(6):2217-2223.

Table E3. Remodeling in bronchial biopsies

			Controlled Asthma	**Uncontrolled Asthma**
		Totally controlled (n=19)	Well controlled (n=33)	Uncontrolled (n=37)
Epithelium	Goblet cells (N)	37 (4-85)	35 (2-92)	39 (9-119)
	Mucus (%)	8 (0.8-21.2)	13 (0.9-33.9)	9.4 (0.2-46.1)
	Intact epithelium (%)	13 (0-32.8)	10.5 (0-65.3)	5.6 (0-47.6)*
	Basal epithelium (%)	37.2 (14.7-74.3)	40.9 (5.1-80.2)	42.5 (18.5-72.2)
	Denuded BM (%)	16 (0-58.8)	9.6 (0.7-47.6)	17.5 (2.1-63.2)
	Metaplasia (%)	0 (0-26.2)	0 (0-18.2)	0 (0-19.4)
	Thickness (µm)	17.3 (11.2-36.8)	20.4 (7.5-91.4)	17.6 (7.9-48.1)
	$Ki67^+$ (% intact)	5.1 (0.4-11.2)	4.4 (0.9-20.1)	4.0 (0-15.5)
	$Ki67^+$ (% basal)	2.7 (0-8.5)	5.1 (0.4-28.9)	3.8 (0-25.5)
Submucosa	Collagen 3 (%)	45.4 (8.2-64.5)	39 (8.6-77.9)	42 (15.5-64.9)

Values are medians (ranges). N number; * $p<0.05$ versus control.