ONLINE SUPPLEMENT

Detailed methods

Subjects

The original cohort of the Asthma Management Protocol University Leiden (AMPUL) study consisted of 75 atopic patients with mild-to-moderate persistent asthma,[1] of whom 45 patients with successful bronchoscopy at baseline (t=0) were included in the study. At inclusion patients were atopic, 18-50 years, non- or ex-smokers (<5 pack years), experienced symptoms of episodic chest tightness and wheezing, and 77% of the patients were using inhaled steroids on a regular base. Patients with a pre-bronchodilator (pre BD) FEV_1 higher than 50% predicted and post-bronchodilator (post BD) FEV_1 higher that 80% predicted were included. All patients were hyperresponsive (provocative concentration of methacholine causing a fall of 20% in FEV_1 [PC_{20}] <8 mg/ml).

Amongst the 45 patients who underwent a successful bronchoscopy at baseline (t=0), 37 underwent a bronchoscopy at 2 years of follow-up (t=2).[1] Subsequently, 32 patients had a clinical assessment at 7.5 years of follow-up (t=7.5).[2] Amongst those, 19 patients underwent spirometry and bronchoscopy at 14 years of follow-up (t=14; current study). Patients who were lost to follow-up or did not participate in the current study did not differ in FEV_1, PC_{20} or steroid use from those who participated ($p>0.10$; data not shown). A flow chart of patient numbers during this 14 year follow-up study is given in Figure 1

Design

This was a prospective, observational study covering 14 years of follow-up. Pre- and post-bronchodilator (BD) FEV_1, the latter as a measure of non-reversible airways obstruction, and PC_{20}, were measured at baseline and t=14 years. Bronchoscopy was performed at baseline and

t=14 years. During the first 2 years, patients were treated according to either existing guidelines or a treatment strategy aimed at reducing airway responsiveness whilst treatment was adjusted by a chest physician every 3 months.[1] Between 2 and 14 years of follow-up patients were treated for asthma, if necessary, by their own general practitioner or chest physician.

Spirometry and Airway Responsiveness

Spirometry was performed according to the same procedures throughout the study.[1] Patients withheld use of short-acting β_2-agonists for 8 hours and long-acting β_2-agonists for at least 24 hours before the measurements, and smoking was ceased for 24 hours. Post BD FEV_1 was measured 15 minutes after inhalation of 400 µg salbutamol. Airway hyperresponsiveness was determined using methacholine and was expressed as PC_{20}.

Bronchoscopy and immunohistochemistry

At t=0 and t=14 bronchial biopsies were obtained from right lower lobe subsegments, the middle lobe, and carina. Detailed bronchoscopic and immunohistochemistry methods for baseline have been published previously.[1] Bronchoscopies were performed by an experienced pulmonary physician (L.N.A.W.) using a fiberoptic bronchoscope (Exera II CV-180, Olympus, Hamburg, Germany) and pairs of cup forceps (Radial Jaw 3, Boston Scientific, Natick, MA, USA). Patients received the following premedication: inhalation of 400 µg salbutamol, and local anaesthesia with lidocaine (1% solution intranasally, 10% pharyngeal spray, 1% solution via the bronchoscope for trachea and bronchi).

At baseline, bronchial biopsies were analysed for reticular layer thickness, eosinophilic cationic protein (EG2), mast cell tryptase (AA1), CD3, CD4 and CD8. Detailed biopsy

methods have been published previously.[1] At t=14, biopsy tissue was fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 24 hours, and embedded in paraffin. Paraffin embedded biopsies were cut in 4 µm thick sections and Haematoxylin- and eosin staining (H&E) was used for evaluation and selection of the four morphological best biopsies per patient for analysis (without crushing artifacts, large blood clots, or only epithelial scrapings). For all immunostainings, endogenous peroxidase activity in the sections was blocked in 0.3% hydrogen peroxide in methanol. After antigen retrieval, the following stainings were performed: EG2, AA1, elastase (NE), , CD4, CD8, -CD68, CD20, DC-SIGN, granzyme B, and Ki67. Periodic acid-Schiff/Alcian Blue (PAS/AB) staining was used for mucin detection. Antibodies were diluted in 1% bovine serum albumin (BSA) in PBS. PBS/BSA 1% alone was used as a negative control for the stainings. For all immunohistochemical stainings, EnVision System® anti-mouse horseradish peroxidase (HRP) (Dako-Corporation, Carpinteria, USA) was used as secondary antibody and stainings were visualized with EnVision System® NovaRed (Dako-Corporation) as a chromogen. Details on antibodies and antigen retrieval methods used are shown in Table 1.

Analysis of stainings in bronchial biopsies

Total biopsy images were taken of all sections stained. The length of the basement membrane (BM) was determined of both intact (A) and damaged epithelium (B) in PAS/AB-stained sections, and was expressed as % damaged epithelium of total epithelium ((B/(A+B))*100). The length of the basement membrane was measured underneath both intact as well as damaged epithelium. Intact epithelium was defined as a layer of both basal and columnar cells attached to the basement membrane, including areas of goblet cell hyperplasia or metaplasia. Damaged epithelium was defined as the remainder of the epithelium, including denuded basement membrane.[3, 4] Ki67 was analysed by a fully automated procedure[5] in intact

epithelium. Results were expressed as the number of $Ki67^+$ cells per millimeter BM. PAS/AB^+- area in intact epithelium was analysed by densitometry and was expressed as % PAS/AB^+ area.

The number of positive stained cells for EG2, AA1, NE, CD3, CD4, CD8, CD68, CD20, DC-SIGN and Ki67 was counted by a validated full automated procedure[5] (KS400; Carl Zeiss B.V., Sliedrecht, The Netherlands). Data were expressed as number of positive cells per 0.1 mm^2 lamina propria. Since very few cells were stained for granzyme B, a semi-quantitative scoring method instead of automated computerized analysis was used. Granzyme B staining was analysed semi-quantitatively by two blinded observers, and was expressed as fraction of biopsies that contained granzyme B^+-cells.

Statistical analysis

Annual declines in pre- and post BD FEV_1 (in ml/year and % predicted/year) were determined between t=0 and t=14 years ((FEV_1 at t = 14 years - FEV_1 at baseline)/14). The mean (declines in) pre- and post BD FEV_1 were compared using a paired t-test. $EG2^+$-, $AA1^+$-, $CD3^+$-, $CD4^+$-, $CD8^+$-, NE^+-, $CD68^+$-, $CD20^+$-, DC-$SIGN^+$-, and $Ki67^+$- cell counts, PAS/AB^+ area and reticular layer thickness were dichotomized on the basis of median values of the stainings: < median ("low") and ≥ median ("high"). For granzyme B, the difference in (decline in) lung function was analysed between high granzyme B (> 0.5 fraction of positive biopsies) and low granzyme B (<0.5 fraction positive biopsies). Decline in pre and post BD FEV_1 % predicted was compared between subjects with low and subjects with high inflammatory (cell) counts with nonparametric Mann Whitney-U testing. In addition, correlations between $EG2^+$-, $AA1^+$-, $CD3^+$-, $CD4^+$-, $CD8^+$-, NE^+-, $CD68^+$-, $CD20^+$-, DC-$SIGN^+$-,and $Ki67^+$- cell counts, PAS/AB^+ area and reticular layer thickness, and decline of pre and post BD FEV_1% predicted were analysed by nonparametric Spearman's correlation

analysis. For both dichotomous and continuous Spearman's correlation analyses, differences at p-values smaller than 0.05 were considered to be significant.

Cross-sectional associations between the T-cell markers CD3, CD4 and CD8 at t=14 years and pre- and post BD FEV_1 % predicted at t=14 years and associations between $CD3^+$-, $CD4^+$- and $CD8^+$-cell counts at baseline and after 14 years of follow-up were analysed by nonparametric Spearman's correlation analysis.

Statistical analysis was performed with SPSS 16.0 software (Chicago, IL). Differences and correlations with a p-value smaller than 0.05 were considered to be statistically significant.

Sample size estimation on the baseline data showed that the current patient number available at 14 years of follow-up is sufficient for assessment of (differences in) bronchial biopsy inflammatory cell counts (α=0.05 (two-sided) , β=0.20 (one-sided)).[6]

Online Supplement Table 1

TABLE 1. Antibodies used for immunohistochemistry

Antibody against	Species	Clone	Pretreatment*	Dilution	Manufacturer
Eosinophilic cationic protein	mouse	EG2	Trypsin 0.1%	1:600	Pharmacia, Uppsala, Sweden
Mast cell tryptase	mouse	AA1	pH 6	1:10000	Dako, Glostrup, Denmark
Neutrophil elastase	mouse	NP57	-	1:200	Dako, Glostrup, Denmark
CD3	mouse	F7.2.38	pH 9	1:100	Dako, Glostrup, Denmark
CD4	mouse	4B12	pH 6	1:20	MBL, Nagoya, Japan
CD8	mouse	C8/144B	pH 9	1:500	Dako, Glostrup, Denmark
CD20	mouse	L2b	pH 6	1:100	Dako, Glostrup, Denmark
CD68	mouse	PG-M1	pH 6	1:250	Dako, Glostrup, Denmark
DC-SIGN	mouse	19F7	pH 9	1:250	HBT, Plymouth Meeting, PA, USA
Granzyme B	mouse	GrB-7	pH 6	1:50	Sanbio BV, Uden, The Netherlands
Ki67	mouse	MIB-1	pH 6	1:100	Dako, Glostrup, Denmark

*pH 6 = Target Retrieval Solution pH 6 (Dako, Glostrup, Denmark)
pH 9 = Target Retrieval Solution pH 9 (Dako, Glostrup, Denmark)

Online Supplement Table 2

TABLE 2. Results of linear regression analysis with covariates smoking and use of corticosteroids (cs). Slopes (B) of regression lines and p-values are given. This table illustrates that the decline in FEV_1 pre or post % predicted cannot be explained by smoking and use of corticosteroids as potential confounding factors, since the slopes (B) are only slightly affected by including these factors in the analysis.

	Decline pre % predicted		**Decline post % predicted**	
	without covariates	**+ smoking/cs**	**without covariates**	**+ smoking/cs**
CD8, t=0	B= -4.25, p=0.09	B= -3.73, p=0.18	B= -3.79, p=0.02	B= -3.71, p=0.04
CD8, t=14	B= -0.63, p=0.00	B= -0.67, p=0.00	B= -0.28, p=0.05	B= -0.24, p=0.17
CD4, t=0	B= -3.16, p=0.02	B= -2.78, p=0.06	B= -2.36, p=0.07	B= -2.25, p=0.02
CD4, t=14	B= -0.08, p=0.50	B= -0.08, p=0.48	B= -0.01, p=0.86	B= -0.04, p=0.61
CD3, t=0	B= -2.99, p=0.27	B= -2.41, p=0.39	B= -1.64, p=0.38	B= -1.45, p=0.43
CD3, t=14	B= -0.15, p=0.03	B= -0.15, p=0.08	B= -0.04, p=0.43	B= -0.03, p=0.58

Online Supplement References

1 Sont JK, Willems LN, Bel EH, Van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am J Respir Crit Care Med 1999; 159: 1043-51.

2 van Rensen EL, Sont JK, Evertse CE, Willems LN, Mauad T, Hiemstra PS, Sterk PJ. Bronchial CD8 cell infiltrate and lung function decline in asthma. Am J Respir Crit Care Med 2005; 172: 837-41.

3 Pilette C, Godding V, Kiss R, Delos M, Verbeken E, Decaestecker C, De Paepe K, Vaerman JP, Decramer M, Sibille Y. Reduced epithelial expression of secretory component in small airways correlates with airflow obstruction in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2001; 163: 185-94.

4 Lapperre TS, Sont JK, van Schadewijk A, Gosman MM, Postma DS, Bajema IM, Timens W, Mauad T, Hiemstra PS. Smoking cessation and bronchial epithelial remodelling in COPD: a cross-sectional study. Respir Res 2007; 8: 85.

5 Sont JK, De Boer WI, van Schadewijk WA, Grunberg K, Van Krieken JH, Hiemstra PS, Sterk PJ. Fully automated assessment of inflammatory cell counts and cytokine expression in bronchial tissue. Am J Respir Crit Care Med 2003; 167: 1496-503.

6 Sont JK, Willems LN, Evertse CE, Hooijer R, Sterk PJ, Van Krieken JH. Repeatability of measures of inflammatory cell number in bronchial biopsies in atopic asthma. Eur Respir J 1997; 10: 2602-8.