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Airway eosinophilia in remission and progression of asthma: Accumulation with a fast decline of FEV_1

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Summary
Background: As it is unknown whether complete asthma remission or progression of asthma is associated with airway inflammation and remodeling, we assessed these characteristics in bronchial biopsies of relevant subsets of asthma patients.
Methods: Sputum and bronchial biopsies were obtained from asthma patients in remission (PC_{20} histamine $>$ 32 mg/ml, PC_{20} AMP $>$ 320 mg/ml) and from those with either a slow FEV_1 decline ($<$30 ml/year) or fast decline ($>$30 ml/year). Inflammatory cells and mediators were determined in sputum, inflammatory cells and aspects of airway remodeling in bronchial biopsies.
Results: Asthmatics in remission and asthma patients with a slow FEV_1 decline had a similar extent of airway inflammation and remodeling in sputum and bronchial biopsies. Asthma patients with a fast FEV_1 decline had high sputum eosinophil numbers. Moreover, FEV_1 decline (ml/year) correlated with sputum eosinophil numbers ($Rs = 0.51$, $p = 0.003$) and ECP levels ($Rs = 0.57$, $p = 0.001$). Airway remodeling, i.e. basement membrane thickness, correlated with sputum eosinophils ($Rs = 0.69$, $p < 0.001$), sputum ECP ($Rs = 0.46$, $p = 0.018$) and airway wall eosinophil numbers ($Rs = 0.49$, $p = 0.002$).
Conclusions: Asthma, even when in remission, is accompanied by airway inflammation and remodeling. Data suggest that eosinophils are important in a subset of asthma patients by association to accelerated FEV_1 decline and change of basement membrane thickness.

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Introduction

Asthma is a chronic inflammatory disorder of the airways associated with airway obstruction that is often reversible, either spontaneously or with treatment. Nevertheless, asthma patients have on average a lower lung function than healthy controls.[1] Furthermore, some patients have an even accelerated decline in forced expiratory volume in 1 s (FEV_1) over time,[2] probably caused by ongoing airway inflammation and the resulting airway remodeling.[3] Not all asthmatics are predestined for this scenario since some subjects show stable lung function and others even outgrow their disease. As yet, it is unknown whether these differences in the natural course of clinical asthma reflect differences in the underlying inflammatory processes and structural changes in the airways.

Some patients fully outgrow their asthma, so-called asthma remission, which is characterized by normal lung function, absence of bronchial hyperresponsiveness (BHR) and respiratory symptoms while not using asthma medication.[4] Others are in "clinical" asthma remission, i.e. they are asymptomatic but still show BHR. The latter type of remission is associated with ongoing airway inflammation and remodeling as reflected by increased numbers of eosinophils, T-cells and mast cells in the airway mucosa and thickening of the reticular basement membrane (BM) when compared to control subjects without asthma.[5] However, it is unknown whether complete remission with the absence of BHR is associated with less airway inflammation and remodeling when compared to persistent asthma. Therefore, we investigated airway inflammation and remodeling in patients with complete asthma remission, defined by the absence of BHR, asthma symptoms and treatment.

Previous studies have revealed multiple risk factors for an accelerated FEV_1 decline (15–50 ml/year) in asthma: low baseline lung function (FEV_1 % predicted),[6] less reversibility to β2-agonists,[6] more severe BHR,[7] smoking,[8] increased mucus production,[2] male gender[9] and frequent exacerbations.[10] The pathological processes underlying the accelerated FEV_1 decline in asthma are unknown. Several suggestions for the underlying pathological process come from cross-sectional studies demonstrating an association between the above described risk factors and markers of airway inflammation and remodeling. For example, low baseline lung function has been associated with increased airway wall thickness,[11] more severe BHR with thickening of the subepithelial BM,[12] smoking with epithelial metaplasia,[13] goblet cell hyperplasia[14] and increased subepithelial collagen deposition,[15] and increased mucus production has been associated with hyperplasia and hypertrophy of epithelial goblet cells[16] and increased presence of submucosal mucous glands.[16] Moreover, Ulrik et al.[17] showed an association between higher blood eosinophil numbers and accelerated FEV_1 decline in adult asthma patients, thereby postulating a role of eosinophils. Based on these findings it is tempting to speculate that an accelerated FEV_1 decline in asthma indeed results from progressive airway inflammation and remodeling.

Aim of this study was to characterize the pathological background of asthma remission and progression. Therefore, we recruited 47 steroid-naïve, non-smoking subjects with a documented asthma diagnosis in the past, we assessed their annual FEV_1 decline over a minimum period of at least 5 years and categorized them into 3 groups: subjects with asthma remission, current asthma patients with a slow FEV_1 decline (<30 ml/year) and a fast FEV_1 decline (>30 ml/year). Airway inflammation and remodeling were investigated in sputum samples and bronchial biopsies.

Methods

Subjects

Non-smoking subjects (n = 47) aged between 32 and 70 years, without oral or inhaled corticosteroids were recruited. All subjects originated from research cohorts investigated earlier by our research group and all had a doctor's diagnosis of asthma and a documented PC_{20} histamine (using 30-s tidal breathing) ≤ 32 mg/ml in the past.[4] These subjects were extensively characterized according to a standard protocol (including FEV_1) at least five years prior to our study. Subjects were re-assessed in the present study with lung function, hyperresponsiveness to adenosine 5′-monophosphate (AMP), sputum induction and bronchial biopsies. Three groups were identified: 1) asthma remission (FEV_1 > 90% predicted, PC_{20} AMP > 320 mg/ml, PC_{20} histamine > 32 mg/ml), 2) slow FEV_1 decline (<30 ml/year over at least 5 years and PC_{20} AMP ≤ 320 mg/ml or PC_{20} histamine ≤ 32 mg/ml), 3) fast FEV_1 decline (>30 ml/year over at least 5 years and PC_{20} AMP ≤ 320 mg/ml or PC_{20} histamine ≤ 32 mg/ml). Main exclusion criteria were: the use of corticosteroids during the period over which the FEV_1 decline was being calculated or more than 5 years of cumulative corticosteroid use in the distant past. Furthermore, FEV_1 < 1.2 l, bronchiectases, upper respiratory tract infection (e.g. colds) and/or use of antibiotics the last 2 months before inclusion were exclusion criteria. The study protocol was approved by the local medical ethics committee. All subjects gave their written informed consent.

Study design

Peripheral blood eosinophil counts and IgE, induced sputum samples and lung function (flow-volume, CO diffusion) were obtained. An AMP provocation test was performed followed 1–2 weeks later by a bronchoscopy. Subjects with a negative AMP provocation test (PC_{20} AMP > 320 mg/ml) were subjected to a histamine provocation test >3 weeks later to determine BHR.

Lung function

FEV_1 was measured with a calibrated water-sealed spirometer at the current and past visit (Lode Spirograph D53, Lode Instruments, Groningen, the Netherlands). Reversibility of FEV_1 (% predicted) was measured after administration of 800 μg of Albuterol. Annual FEV_1 decline (ml/year) was calculated by subtracting FEV_1 (post-bronchodilator) at the first and second measurement, divided by

the number of years between the two measurements (minimum of 5 years). Provocation tests were performed with a method adapted from Cockcroft and coworkers.[18] After an initial nebulized 0.9% saline challenge, subjects inhaled doubling concentrations AMP (0.04–320 mg/ml) by 2-min tidal breathing, at 5-min intervals. BHR to histamine was tested as reported previously,[19] using 30-s tidal breathing and doubling concentrations ranging from 0.13 to 32 mg/ml.

Atopy measurements

Atopy was determined by the Phadiatop screening test with the ImmunoCap system (Phadia AB, Sweden) and results are presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). Positive Phadiatop was defined as patient serum/control serum >1.

Sputum induction and processing

Sputum was induced by inhalation of 5% hypertonic saline aerosols as over 3 consecutive periods of 5 min. Processed whole sputum samples were stained with May Grünwald Giemsa (MGG) to obtain cell differentials by counting total 600 viable, non-squamous cells. Sputum was not scored if the percentage squamous cells was $>80\%$ or the total number of non-squamous cells was <600.

Sputum supernatant measurements

Histamine was measured by ELISA (IBL, Hamburg, Germany). Adenosine was measured by HPLC as described previously.[20] The concentration of eosinophilic cationic protein (ECP) was measured using a fluoroenzyme immunoassay (ImmunoCAP ECP, Pharmacia, Uppsala, Sweden).

Secreted mucin was measured by double-sandwich ELISA (Covance, Emeryville, USA), with the 17B1 protein-G purified antibody and 17Q2 alkaline phosphatase-conjugated antibody specifically binding to the granules of the surface goblet cells and mucous gland cells, as previously described.[21]

Collection, processing and immunohistochemical staining of bronchial biopsies

After local anaesthesia, bronchial biopsies were obtained using a flexible bronchoscope (type Olympus BF P20 or BF XT20) from segmental divisions of the main bronchi. The biopsies were fixed in 4% formalin, processed and embedded in paraffin. Bronchial biopsies were cut in 3 μm thick sections. All stainings were performed in an automated system using the DAKO autostainer and were manually counterstained with methylgreen.

The inflammatory profile was assessed with specific antibodies against eosinophilic peroxidase (EPX, the laboratories of Drs. NA Lee and JJ Lee), 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 and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) except for EPX and CD8, which were detected using biotinylated anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL, USA) and alkaline phosphatase- (DAKO) or peroxidase-labelled streptavidine conjugates (DAKO). Permanent Red (DAKO) or NovaRED (Vector Labs, Burlingame, USA) was used as chromogen.

Periodic acid-Schiff (PAS) staining was used to determine the presence of goblet cells in the bronchial epithelium. Haematoxylin eosin (HE) staining was used for evaluation of epithelial integrity and basement membrane (BM) thickness.

Deposition of extracellular matrix (ECM) components in the subepithelial compartment was assessed by specific antibodies against collagen 3 (Southern Biotechnology), decorin (dermatan sulfate proteoglycan, Seikagaku, Tokyo, Japan) and versican (large proteoglycan (versican), Seikagaku). In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) except for collagen 3, which was detected using peroxidase-labelled rabbit anti-goat and goat anti-rabbit conjugates (both DAKO). 3.3′-Diaminobenzidine tetrachloride (DAKO) was used as chromogen.

Quantification of the histological stainings

Quantification of inflammatory cells was performed using computerized-assisted image analysis at a magnification of 200× (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). A blinded investigator counted the number of positively stained inflammatory cells in the submucosal area. This submucosal area was restricted to 100 μm under the basement membrane (BM) and a total area of 0.1 mm^2. For each subject three sections of one biopsy sample were counted (totally 0.3 mm^2 tissue) and average numbers were used for further analysis.

The number of goblet cells was counted on PAS-stained biopsy sections, in layers with intact epithelium, and expressed per mm of BM. Epithelial integrity was determined using HE-stained biopsy sections and was expressed as the percentage of BM that was covered with either intact epithelium (a layer of basal and ciliated columnar epithelial cells without detachment from the BM), only basal epithelial cells, metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells) or the percentage of BM that was denuded from epithelial cells. BM thickness was determined by the measurement of the total BM length and surface on HE-stained biopsy sections. Tangentially cut sections were excluded. Subsequently, the mean thickness could be calculated by dividing the BM surface area by its length.

Expression of ECM components was measured by determining the colour threshold of stained tissue, using computerized-assisted image analysis. The percentage of positive tissue per biopsy section was determined in the submucosal area 200 μm under the BM. For each subject two different biopsy sections were quantified and average percentages were used for further analysis.

Statistics

All analyses were performed using SPSS (version 14.0; SPSS Inc, Chicago, IL, USA). Normality of distributions was assessed using the Kolmogorov–Smirnov test. When necessary, normalization by transformation was attempted. Normally distributed data was analysed with one-way ANOVA and independent samples t-tests. Non-normally distributed data was analysed with Kruskal–Wallis and Mann–Whitney U tests. Chi-square tests were used to compare groups for dichotomous variables. Correlations were evaluated by Pearson (for normally distributed data) or Spearman (for non-normally distributed data) tests. Two tailed p-values of <0.05 were considered statistically significant.

Results

Subject characteristics

Forty-seven subjects were included: 10 subjects with complete asthma remission, 20 asthma patients with a slow FEV_1 decline over at least 5 years (<30 ml/year) and 17 with a fast FEV_1 decline over at least 5 years (>30 ml/year). As expected, FEV_1 was lowest in asthmatics with a fast FEV_1 decline, intermediate with a slow decline and highest in asthma remission; reversibility was not significantly different between the three groups. Asthma patients with a fast FEV_1 decline had significantly higher blood eosinophil counts than those with a slow FEV_1 decline (Table 1).

Inflammatory cells and inflammatory mediators in sputum

The absolute number of sputum eosinophils was higher in patients with a fast FEV_1 decline than with a slow FEV_1 decline (Table 2, Fig. 1A). Annual FEV_1 decline correlated significantly with the absolute number of sputum eosinophils (Fig. 1B). Sputum neutrophils, macrophages and lymphocytes did not significantly differ between the three study groups (Table 2).

The levels of inflammatory mediators in the supernatant of induced sputum, i.e. histamine, adenosine and mucin were similar for subjects with asthma remission and asthma patients with a slow or a fast FEV_1 decline (Table 2). Moreover, the levels of inflammatory cytokines and growth factors (IFN-γ, IL-4, 5, 8, 13, 17, EGF, Eotaxin, FGFb, GM-CSF, MCP1, RANTES, VEGF) were similar for all groups (See additional file 1). Sputum ECP values were highest in asthma patients with a fast FEV_1 decline (59.8 (12–2467) μg/l), values being significantly higher than in subjects with asthma remission (16.0 (3–172) μg/l) and slightly, but not significantly, higher than in asthma patients with a slow FEV_1 decline (27.0 (6–447) μg/l, $p = 0.056$) (Table 2, Fig. 1C). Moreover, a higher annual FEV_1 decline correlated significantly with higher sputum ECP levels (Fig. 1D).

Inflammatory cells in bronchial biopsies

No significant differences were observed in numbers of eosinophils, mast cells, macrophages, neutrophils and T-lymphocytes in bronchial biopsies of subjects with asthma remission, asthma patients with a slow or fast FEV_1 decline (Table 3, Fig. 2A–C).

Airway remodeling determined in bronchial biopsies

No differences were observed in remodeling parameters in bronchial epithelium (goblet cells and epithelial integrity), BM (thickness) and submucosal tissue (presence of ECM components) between subjects with asthma remission and asthma patients with a slow or fast FEV_1 decline (Table 4).

Table 1 Patient characteristics.

	Remission (n = 10)	Slow FEV_1 decline (n = 20)	Fast FEV_1 decline (n = 17)
Sex (M/F)	3/7	11/9	12/5
Age (yr)	53 (32–67)	48 (35–70)	53 (38–64)
FEV_1 decline (ml/year)	8.0 (−35–35)	18.6 (−27–29)	50.3 (34–105)$^{\#,\$}$
Interval between FEV_1 measurements (years)	8.7 (6–14)	8.9 (5–13)	9.3 (5–13)
FEV_1 (% predicted)	111 (102–146)	100 (78–131)*	98 (77–127)$^{\#}$
FEV_1/VC (%)	79 (67–93)	75 (65–91)	73 (56–84)$^{\#}$
MEF_{50} (% predicted)	105 (60–147)	69 (46–147)*	68 (36–98)$^{\#}$
Reversibility FEV_1 (%)	3.9 (0.4–11.5)	8 (−0.4–28.7)	8.3 (−8.4–17.2)
PC_{20}AMP (mg/ml)	>320	>320 (0.02 to >320)*	46.2 (0.02 to >320)$^{\#}$
KCO (% predicted)	95 (84–131)	99 (78–127)	110 (82–141)
IgE (IU/L)	60 (7–231)	66 (14–552)	70 (11–558)
Positive Phadiatop (n (%))	5 (50%)	13 (65%)	13 (76%)
Phadiatop score (ratio)	0.96 (0.33–54)	9.1 (0.22–92)	15 (0.06–92)
Blood eosinophils ($\times 10^9$/l)	0.12 (0.06–0.24)	0.12 (0.02–0.35)	**0.19 (0.07–0.51)$^{\$}$**

Values are presented as median values (ranges), unless stated otherwise. All spirometric data were determined after inhalation of 800 μg of Albuterol; reversibility FEV_1 = change in FEV_1, expressed as increase in percentage predicted value after 800 μg of Albuterol; PC_{20} AMP = provocative concentration of adenosine 5′ monophoshate causing a 20% fall in FEV_1; Positive Phadiatop = specific IgE's in patient serum/control serum >1; n = number. *$p < 0.05$ remission vs. slow FEV_1 decline, $^{\#}p < 0.05$ remission vs. fast FEV_1 decline, $^{\$}p < 0.05$ slow vs. fast FEV_1 decline.

Table 2 Inflammatory cells and mediators in sputum.

	Remission (n = 10)	Slow FEV_1 decline (n = 20)	Fast FEV_1 decline (n = 17)
No sputum	2 (20%)	3 (15%)	3 (18%)
Sputum not evaluable			
>80% squamous n (%)	1 (10%)	2 (10%)	3 (18%)
<600 cells n (%)	–	–	2 (11%)
Total cells ($\times 10^6$/ml)	0.46 (0.06–2.90)	0.31 (0.12–2.27)	0.41 (0.03–2.05)
Neutrophils (%) ($\times 10^6$/ml)	62 (19–78)	60 (20–94)	55 (35–85)
	0.40 (0.03–1.19)	0.21 (0.03–1.58)	0.28 (0.11–1.44)
Macrophages (%) ($\times 10^6$/ml)	36 (20–78)	32 (5–76)	33 (13–62)
	0.16 (0.02–2.14)	0.10 (0.01–1.07)	0.18 (0.08–0.61)
Eosinophils (%) ($\times 10^6$/ml)	0.2 (0–4.6)	0.2 (0–4.8)	1.8 (0–26.0)
	0.002 (0–0.03)	0.001 (0–0.02)	**0.01 (0–0.34)***
Lymphocytes (%) ($\times 10^6$/ml)	2.2 (1.4–7.3)	2.6 (0.2–6.9)	1.3 (0–5.6)
	0.02 (0.001–0.04)	0.01 (0.001–0.07)	0.004 (0–0.11)
Histamine (ng/ml)	13.4 (2.8–29.8)	9.1 (0.2–28.8)	13.8 (4.8–38.0)
Adenosine (ng/ml)	56.5 (8.0–110)	34.6 (6.2–178)	23.0 (0.9–201)
ECP (μg/l)	16.0 (2.5–172)	27.0 (6.0–447)	**59.8 (11.9–2467)*,#**
Mucin (units)	0.25 (0.04–3.9)	0.78 (0.08–10.3)	1.0 (0.18–2.7)

Values are presented as median values (percentages or ranges). Eosinophils: *$p < 0.05$ vs. slow FEV_1 decline. ECP: *$p = 0.056$ vs. slow FEV_1 decline, #$p < 0.05$ vs. remission. For luminex data see Table S1.

BM thickness correlated significantly with all investigated parameters in sputum and bronchial biopsies that are associated with eosinophilic inflammation, i.e. sputum eosinophils (Fig. 3A), sputum ECP (Fig. 3B) and EPX^+ cells in biopsies (Fig. 3C). The correlation scatters showed two outliers for BM thickness (Fig. 3). However, without these two subjects, significant correlations were still present between BM thickness and all investigated eosinophil markers (not shown).

Discussion

The present study describes the pathological background of asthma remission and progression in a well-characterized

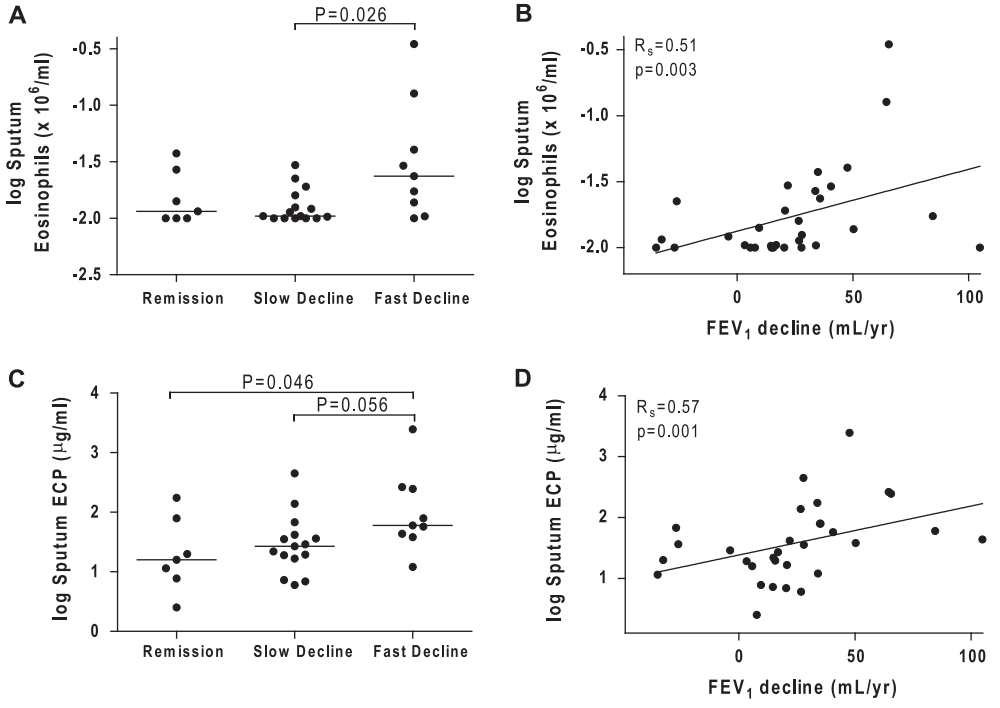

Figure 1 Eosinophilic inflammation in sputum. (A) Absolute number of sputum eosinophils (log transformed) in subjects with asthma remission and asthmatic subjects with a slow or a fast FEV_1 decline. (B) Correlation between the annual FEV_1 decline and the absolute number of sputum eosinophils (log transformed). (C) Sputum ECP (log transformed) in subjects with asthma remission and asthmatic subjects with a slow or a fast FEV_1 decline. (D) Correlation between the annual FEV_1 decline and sputum ECP (log transformed). Each closed circle represents one subject. Horizontal bars represent median values.

Table 3 Inflammatory cells in bronchial biopsies.

	Remission (n = 9)	Slow FEV_1 decline (n = 18)	Fast FEV_1 decline (n = 11)
EPX^+ eosinophils	4 (0–10)	3 (0–18)	3 (0–40)
$AA1^+$ mast cells	5 (1–20)	3 (0–13)	7 (1–19)
$CD68^+$ macrophages	17 (12–86)	16 (4–37)	17 (3–30)
$NP57^+$ neutrophils	9 (2–30)	9 (1–46)	9 (2–38)
$CD3^+$ lymphocytes	75 (35–291)	77 (21–177)	62 (33–246)
$CD4^+$ lymphocytes	19 (9–168)	21 (2–67)	24 (9–65)
$CD8^+$ lymphocytes	28 (7–112)	21 (1–112)	17 (3–103)

Number of cells expressed per 0.1 mm^2. Values are presented as median values (ranges). No significant differences between the 3 groups.

group of asthma patients. We investigated patients with complete asthma remission, i.e. individuals with established asthma in the past who at the present time of investigation lacked asthma symptoms, airway obstruction and bronchial hyperresponsiveness (BHR) while not using any asthma treatment. This remission was associated with a similar extent of inflammation and remodeling as present in individuals with current asthma and an FEV_1 decline in the range of the normal population. In contrast, patients with current asthma and a fast FEV_1 decline had more pronounced eosinophilic inflammation in blood and sputum. In line with this observation, the annual FEV_1 decline correlated significantly with eosinophilia in sputum. Basement membrane (BM) thickness, which is considered as an important hallmark of airway remodeling, was positively correlated with eosinophilic inflammation in both sputum and the airway wall.

An important finding of our study is that these patients with complete asthma remission have signs of airway wall inflammation and remodeling. To our surprise, the extent of airway inflammation and remodeling is compatible with that of asthma patients with a slow FEV_1 decline. The question remains how to explain the similar findings in these two different groups of asthma patients. One possible explanation is that the clinical disease activity is not determined by the number but rather the activation state of inflammatory cells, and that complete asthma remission does not excludes the presence of quiescent inflammatory cells. However, our data shows that sputum levels of inflammatory cytokines and growth factors were similar in subjects with remission and current asthma, suggesting that the activation status and functionality of the inflammatory cells is not different. Another explanation may be that clinical disease activity is determined by inflammation and/or remodeling in other regions of the lung not accessible by induced sputum or bronchial biopsies. The type of clinical expression may for example be caused by the volume of smooth muscle in more distal parts of the central or peripheral airways, or the presence or absence of activated mast cells between smooth muscle bundles.

Our findings extend observations in other studies since ongoing airway inflammation and remodeling were also reported in subjects with less strict criteria for asthma remission,[5,22] i.e. with BHR still present. It is still possible that these types of asthma patients need anti-inflammatory treatment. This is an intriguing yet unresolved question since no study has prospectively followed these patients to older age.

Our second interesting observation is that an accelerated FEV_1 decline is associated with increased sputum eosinophil numbers and ECP concentrations. This finding extends results of several cross-sectional studies demonstrating the relationship between eosinophilic inflammation and airway obstruction in asthma[23,24] and a longitudinal study showing a positive association between blood eosinophils and FEV_1 decline in asthma.[17] However, FEV_1 decline in our asthma patients was not associated with the number of airway wall eosinophils, just as van Rensen et al.[25] found. There are two possible explanations for this discrepancy. First, bronchial biopsies give only a snap-shot of the cellular composition of the airway wall which is not identical to the traffic of inflammatory cells through the airway wall. Second, an accelerated FEV_1 decline may be

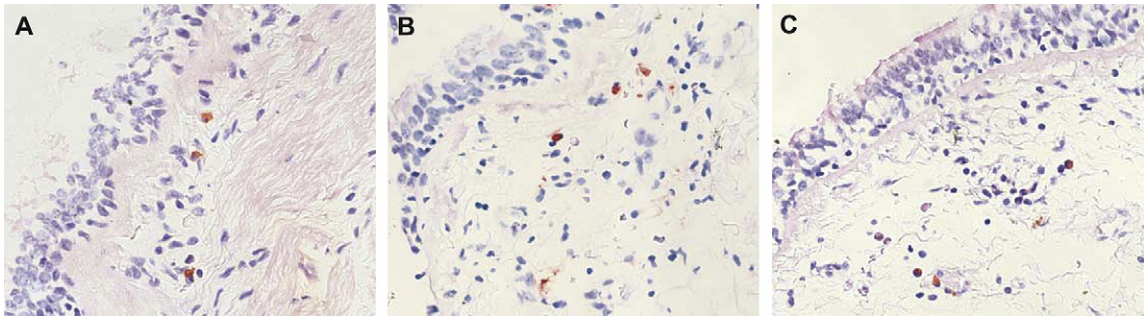

Figure 2 EPX^+ eosinophils in bronchial biopsies. Representative pictures of EPX^+ eosinophils in biopsies of subjects in (A) asthma remission and asthmatic patients with a (B) slow FEV_1 decline or a (C) fast FEV_1 decline. Lens magnification 400×.

Table 4 Remodeling in bronchial biopsies.

		Remission (n = 9)	Slow FEV_1 decline (n = 18)	Fast FEV_1 decline (n = 11)
Epithelium	Goblet cells (N)	28 (0–42)	36 (7–78)	22 (4–220)
	Intact epithelium (%)	6.8 (0–32)	6.8 (0–30)	6.7 (0–34)
	Basal epithelium (%)	63 (30–82)	59 (21–100)	50 (13–86)
	Denuded BM (%)	22 (0–48)	8 (0–52)	13 (0–59)
	Metaplasia (%)	0 (0–21)	0 (0–26)	0 (0–19)
Basement membrane	BM thickness (μm)	6.3 (4.9–8.4)	6.6 (3.8–8.6)	7 (4.4–12.6)
Submucosa	Versican (%)	45 (20–67)	38 (19–70)	44 (29–69)
	Collagen 3 (%)	39 (15–48)	36 (17–63)	40 (9–65)
	Decorin (%)	43 (16–76)	36 (15–85)	44 (27–55)

Values are presented as median values (ranges). Intact and basal epithelium, denuded basement membrane (BM) and metaplasia are presented as the percentage of BM covered with this type of epithelium. Versican, collagen 3 and decorin are presented as the percentage of positive tissue. No significant differences between the 3 groups.

associated with increased recruitment of eosinophils from the bone marrow to the blood. Possibly, these eosinophils move straight from the blood to the luminal space of the lung and are not retained in the airway wall e.g. due to epithelial damage.

Although not demonstrated by our study, it can be anticipated that additional aspects of inflammation, besides eosinophils, contribute to the FEV_1 decline in asthma as well. Previous studies already have shown an association between lower FEV_1 levels and neutrophilic inflammation in sputum[26] and between accelerated FEV_1 decline and CD8-positive T-cells in bronchial biopsies.[25] However, these studies did, in contrast to the "clean population" in our study, include asthma patients who smoked and/or used ICS, and it is known that smoking and ICS use change the inflammatory profile.[19,27]

Our study did not find significant associations between FEV_1 decline and different remodeling parameters like mucus secretion in sputum and goblet cells in bronchial epithelium. Lange et al. reported an association between self-reported mucus production and FEV_1 decline.[2] The fact that the latter study was a large epidemiological study over many years including a heterogeneous group of smoking and non-smoking asthmatics may explain the difference with our study.

Thickening of the subepithelial BM as a result of ECM deposition is one of the hallmarks of airway remodeling[28] and was previously shown to be negatively correlated

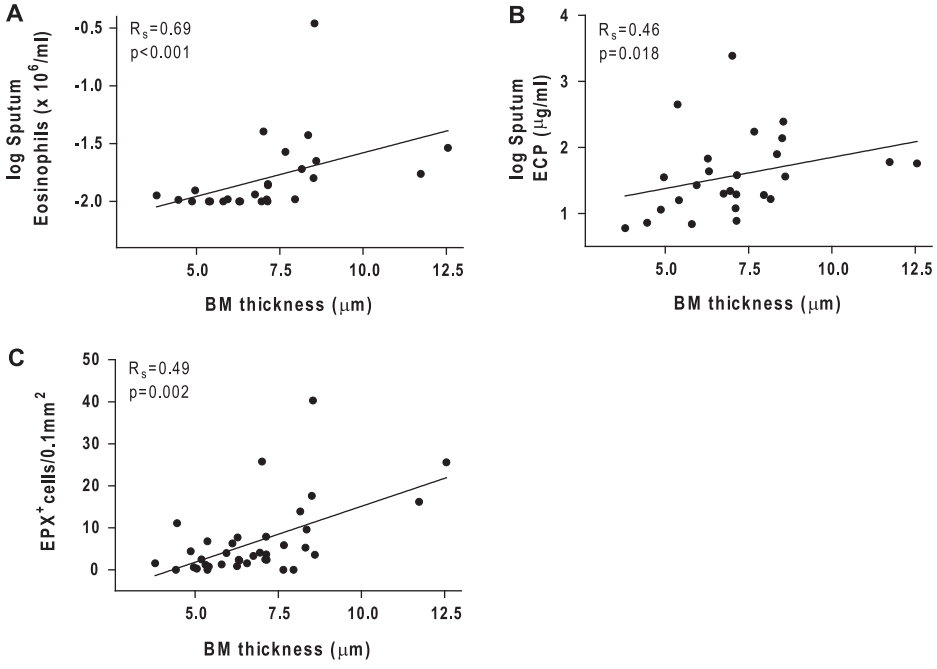

Figure 3 Eosinophilic inflammation and basement membrane thickness. (A) Correlation between basement membrane (BM) thickness and the absolute number of sputum eosinophils (log transformed). (B) Correlation between BM thickness and sputum ECP (log transformed). (C) Correlation between BM thickness and the number of EPX^+ cells in 0.1 mm^2 bronchial biopsy tissue. Each closed circle represents one subject.

with the level of FEV_1 in asthma.[29] We did not observe an association between BM thickness and the annual FEV_1 decline, which is in line with observations by van Rensen et al.[25] It has to be realized that BM thickening in asthma is considered to constitute an early airway wall change, already observed in childhood asthma.[30] This may possibly explain why we did not observe an association with FEV_1 decline over the last years. Considering the limited thickening of the BM, mainly in main bronchi, and all functional studies until now, we think it is less likely that BM thickening contributes to airway dynamics.

Importantly, BM thickness correlated significantly with sputum eosinophils, ECP concentrations in sputum and eosinophils in bronchial biopsies, but not with FEV_1. Indeed airway eosinophils have been shown to produce TGF-β[31] whereas ECP stimulates secretion of TGF-β by human lung fibroblasts.[32] TGF-β is a well-established inducer of ECM production by stimulating the proliferation and differentiation of ECM producing cells, i.e. (myo)fibroblasts.[33] This can lead to excessive ECM deposition at the lamina reticularis and thus thickening of the airway subepithelial BM. So far it is unclear whether thickening of the BM is an epiphenomenon or really contributes to the accelerated FEV_1 decline.

We realize that our study has both strengths and weaknesses. A strength is that we defined asthma remission by the additional criterion of the absence of BHR for both AMP and histamine. Thus our definition of asthma remission is more strict than the classical description of asthma remission,[5,22] and it is better distinguishable from current asthma. Another strength is that we carefully excluded subjects with a history of smoking and use of (oral or) inhaled corticosteroids since ICS are known to reduce[27] and smoking to accelerate FEV_1 decline in asthma.[19] The consequence of this exclusion may be that we included less severe asthma patients, thereby underestimating the differences in inflammation and remodeling. However, the obvious advantage of this exclusion is that our population enabled us to study the natural course of clinical asthma without these important confounding factors. A relative weakness of our study is that, although we studied FEV_1 longitudinally in our patients, we only sampled sputum and bronchial biopsies at the end of follow-up. Therefore, we cannot exclude the possibility that eosinophilic inflammation in sputum is not the cause but rather the consequence of accelerated FEV_1 decline. Second, it can be argued that for an accurate estimation of longitudinal decline in lung function, two measurements, with an average interval of 8.9 years, are not sufficient.[34] However, we would like to emphasize that the two spirometric measurements in our study were performed according to a standardized protocol using the same equipment.

The present study is of clinical relevance since it suggests the need for specific adaptations in therapy in subsets of asthma patients. First, we showed that subjects with complete asthma remission have airway inflammation. This observation does not support but also not refutes the recommendation to stop the use of ICS in these subjects. Long-term studies may give a definite answer on the question whether airway inflammation ultimately disappears completely. Second, we showed that asthma patients with a fast FEV_1 decline are characterized by increased eosinophilic inflammation in blood and sputum. Therefore, intensive ICS treatment, known to reduce the accelerated FEV_1 decline,[27] might be especially beneficial for asthmatics with increased eosinophilic inflammation. Unfortunately, a subset of asthmatics with increased eosinophilic inflammation does not respond to intensive ICS treatment, e.g. due to cigarette smoking, concomitant neutrophilic inflammation, or small airways involvement. In this specific subset of asthmatics other measures and medicaments should be considered.

In conclusion, complete clinical and functional asthma remission is accompanied by airway inflammation and remodeling. This indicates that there is no strong relationship between airway inflammation and BHR and, more importantly, that asthma remission never is really "complete". Furthermore, eosinophilic inflammation may be important in a subset of asthma patients as eosinophils are associated with accelerated FEV_1 decline and increased BM thickness.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rmed.2010.03.030.

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