

Persisting Remodeling and Less Airway Wall Eosinophil Activation in Complete Remission of Asthma

Martine Broekema^{1,2}, Wim Timens¹, Judith M. Vonk³, Franke Volbeda^{2,4}, Monique E. Lodewijk^{1,2}, Machteld N. Hylkema¹, Nick H. T. ten Hacken², and Dirkje S. Postma²

¹Department of Pathology, ²Department of Pulmonology, ³Department of Epidemiology, and ⁴Pulmonary Rehabilitation Center Beatrixoord, University Medical Center Groningen, University of Groningen, The Netherlands

Rationale: Individuals with asthma may outgrow symptoms despite not using treatment, whereas others reach complete remission (CoR) with absence of airway obstruction and bronchial hyperresponsiveness. It is uncertain whether this associates with remission of all inflammatory and remodeling asthma features.

Objectives: To compare the pathologic phenotype of individuals with asthma with CoR and clinical remission (ClinR) and those with active asthma, with and without the use of inhaled corticosteroids (ICS).

Methods: We investigated 165 individuals known with active asthma, on reexamination having CoR (n = 18), ClinR (n = 44), and current asthma (CuA, n = 103, 64 with and 39 without ICS).

Measurements Main Results: Inflammatory cells were measured in blood, induced sputum, and bronchial biopsies; histamine and ECP in sputum; and eosinophilic peroxidase (EPX) immunopositivity and remodeling (epithelial changes, E-cadherin expression, basement membrane [BM] thickening, collagen deposition) in bronchial biopsies. Median (range) blood eosinophils from CoR were significantly lower than those from CuA (0.10 [0.04–0.24] vs. 0.18 [0.02–1.16] $\times 10^9/L$). Bronchial EPX immunopositivity was lower in CoR than in both ClinR and CuA (67 [0.5–462] vs. 95 [8–5329] and 172 [6–5313] pixels). Other inflammatory findings were comparable. BM thickness was lowest in CuA, caused by lower BM thickness in those using ICS (CoR, 6.3 [4.7–8.4]; ClinR, 6.5 [3.8–11.7]; CuA, 5.7 [2.8–12.6]; and ICS using CuA, 5.3 [2.8–8.2] μm).

Conclusions: CoR is still accompanied by airway abnormalities because BM thickness is similar in individuals with asthma with CoR, ClinR, and CuA without ICS. Airway eosinophilic activation best differentiates these three groups, signifying their importance in the clinical expression and severity of bronchial hyperresponsiveness in asthma.

Keywords: airway inflammation; airway remodeling; airway pathology

Some patients with asthma outgrow their disease and reach clinical asthma remission (ClinR) (i.e., their asthma symptoms have disappeared even without using asthma medication, such as inhaled corticosteroids [ICS] and β_2 -agonists). A recent prospective epidemiologic study reported an asthma remission rate of 2 per 100 person-years, asthma remission being defined as no asthma symptoms in 2 consecutive years and no current asthma treatment (1). It is, however, still under debate whether subjects in ClinR have actually outgrown their disease because these subjects may still have (asymptomatic) bronchial hyperresponsiveness (BHR) or a low lung function. Indeed, Boulet

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Individuals with asthma may outgrow symptoms despite not using treatment, whereas others reach complete remission with absence of airway obstruction and bronchial hyperresponsiveness. It is uncertain whether this associates with remission of all inflammatory and remodeling features of asthma.

What This Study Adds to the Field

Asthma can go in full remission according to all clinical phenotypes that characterize asthma, but basement membrane thickening, which accompanies asthma, persists. Importantly, airway wall eosinophilic activation differentiates complete remission from clinical remission and current asthma. This signifies the importance of eosinophil activation for the clinical expression of asthma and the severity of bronchial hyperresponsiveness.

and coworkers (2) have shown that individuals with known asthma yet without current asthma symptoms or asthma treatment for the past 2 years still had subclinical active disease, characterized by BHR, increased reversibility, and low lung function. It is possible that this subclinical active disease reflects ongoing airway inflammation or remodeling. In line with this, higher eosinophil numbers were reported in bronchoalveolar lavage fluid of children with atopy in ClinR (no symptoms for 12 mo and no current medication) compared with healthy controls (3).

There is speculation that the persistence of airway inflammation in ClinR leads to progressive airway remodeling and consequently an increased risk of eventual asthma relapse (3, 4). Indeed, a substantial proportion of subjects in ClinR have a rebound of asthma symptoms in later life (5), suggesting that information provided by the patient on asthma symptoms and medication use is likely insufficient to determine whether asthma is definitively in remission. Therefore, researchers have put forward the term “complete asthma remission” (CoR) signifying the absence of BHR and airway obstruction in addition to the absence of symptoms and asthma treatment (5, 6). One might indeed anticipate that the absence of asthma symptoms, BHR, and airway obstruction in individuals with former asthma who currently do not use asthma treatment is accompanied by normal airway wall pathology (i.e., no inflammation or remodeling).

The aim of the present study was to characterize inflammatory phenotypes in a large group of individuals (n = 165) with CoR (n = 18), ClinR (n = 44), and current asthma (CuA) (n = 103) in airway wall biopsies, induced sputum, and blood. Because these phenotypes can be changed or masked by current ICS treatment, we investigated CuA with (n = 64) and without (n = 39) ICS use in case differences existed between asthma

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Correspondence and requests for reprints should be addressed to Dirkje S. Postma, M.D., Ph.D., Department of Pulmonology, University Medical Center Groningen, Hanzeplein 1, PO Box 30001, 9700 RB Groningen, The Netherlands. E-mail: d.s.postma@long.umcg.nl

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remission and CuA. Thus, we investigated the airway pathology of 165 individuals with known active asthma in the past who, after reexamination, were divided into three groups: (1) CoR; (2) ClinR; and (3) CuA (with and without ICS).

METHODS

Subjects

Subjects with CoR ($n = 18$), ClinR ($n = 44$), and CuA ($n = 103$) were investigated (Table 1). All had a doctor's diagnosis of asthma, and documented reversibility and BHR to histamine in the past. Subjects were reexamined by lung function, BHR to histamine and/or adenosine 5'-monophosphate (AMP), sputum induction, and bronchoscopy with bronchial biopsies. CoR ($n = 18$) was defined as no BHR to histamine (PC_{20} histamine >32 mg/ml) and AMP (PC_{20} AMP >320 mg/ml); normal lung function ($FEV_1 >90\%$ predicted); absence of asthma medication (ICS or β -agonists) use; and absence of asthma symptoms (no wheeze or asthma attacks during the last 3 yr) (5, 6). ClinR ($n = 44$) was defined as absence of asthma medication (ICS or β -agonists) use and absence of asthma symptoms (no wheeze or asthma attacks during the last 3 yr) in patients who formerly had asthma despite the presence of BHR to histamine (PC_{20} histamine >32 mg/ml) or AMP (PC_{20} AMP >320 mg/ml). CuA ($n = 147$) was defined as presence of BHR to histamine (PC_{20} histamine >32 mg/ml) or AMP (PC_{20} AMP >320 mg/ml) and the presence of asthma symptoms (wheeze or asthma attacks during the last 3 yr). The CuA group consisted of 64 patients using ICS and 39 not using ICS. The study protocol was approved by the local medical ethics committee. All subjects gave written informed consent.

Study Design

All subjects were evaluated with blood sampling, sputum induction, and lung function assessment, followed 1 to 2 weeks later by an AMP provocation test. At the third visit, again 1 to 2 weeks later, bronchoscopy with collection of bronchial biopsies was performed. Subjects with a negative AMP provocation test (PC_{20} AMP >320 mg/ml) came for a fourth visit to undergo a histamine provocation test at least 3 weeks later.

Measurements

Lung function, sputum induction, and bronchoscopy with collection of bronchial biopsies were performed as published previously (7) and are described in detail in the online supplement. In short, histamine (IBL, Hamburg, Germany) and neutrophil elastase (Hycult, Uden, The Netherlands) in sputum supernatant were determined by ELISA and eosinophilic cationic protein (ECP) by a fluoroenzyme immunoassay (ImmunoCAP ECP; Phadia AB, Nieuwegein, The Netherlands).

Bronchial biopsies were fixed in 4% formalin, processed and embedded in paraffin, and cut into 3- μ m thick sections. Immunohistochemical staining (except for Ki67 and E-cadherin) were performed using DAKO autostainer (DAKO, Glostrup, Denmark). Immunohistochemistry was performed using antibodies directed against inflammatory cells (*see* online supplement); eosinophilic peroxidase (EPX [8]); mast cell tryptase (AA1, DAKO); proliferation (Ki67, DAKO); epithelial adhesion (E-cadherin; BD Biosciences, Breda, The Netherlands); and collagen 3 (Southern Biotech, Birmingham, AL). Further details on the used immunohistochemistry and quantification procedures are presented in the online supplement.

Statistics

Analyses were performed using SPSS (version 16.0; SPSS, Chicago, IL). Normality of distributions was assessed by Kolmogorov-Smirnov test. If necessary, normalization by transformation was attempted. Normally distributed data were analyzed with one-way analysis of variance and independent sample *t* tests; nonnormally distributed data were analyzed with Kruskal-Wallis and Mann-Whitney U tests. Chi-square tests were used to compare groups for dichotomous variables. Correlations were evaluated by Pearson tests or Spearman tests (normally and nonnormally distributed data [r_s], respectively). Normally distributed data, with or without transformation, were analyzed by multiple linear regression analysis correcting for sex, age, and current smoking. The *P* values of the regression analyses are presented in tables and graphs. Two tailed *P* values of less than 0.05 were considered statistically significant.

Further experimental information can be obtained from the online supplement.

TABLE 1. PATIENT CHARACTERISTICS

	Complete Asthma Remission ($n = 18$)	Clinical Asthma Remission ($n = 44$)	Current Asthma ($n = 103$)
PC_{20} AMP, mg/ml	>320	262 (0.02 to >320)*	38.7 (0 to >320)†‡
FEV_1 , % pred	117 (97–137)	103 (60–123)*	101 (42–135)†
ICS use, n (%)	—	—	64 (62)†‡
ICS dose, μ g/d	—	—	800 (28–2000)
β -Agonist use, n, %	—	—	80 (78)‡
Sex, m/f	7/11	22/22	51/52
Age, yr	48 (17–67)	48 (19–70)	50 (21–71)
FEV_1/VC , %	82 (67–97)	79 (48–97)	73 (39–98)†‡
Reversibility FEV_1 , %	4.8 (–1.7–14.1)	6.2 (–8.4–19.1)	10 (–2.2–38.4)†‡
Atopy, n (%)	10 (56)	24 (55)	76 (74)‡
Phadiatop score, quotient	1.5 (0.2–53.9)	3.9 (0.1–96.5)	13.3 (0.2–128)†‡
Smoking status, n (%)			
current	5 (28)	11 (25)	24 (23)
ex	2 (11)	12 (27)	34 (33)
never	11 (61)	21 (48)	45 (44)
NO alveolar, ppb	4.3 (0.9–12.3)	4.6 (2.1–12.4)	5.6 (1.5–51.7)
NO bronchial, nL/s	0.47 (0.09–1.26)	0.57 (0.24–2.72)	0.80 (0.06–10.38)
Blood eosinophils, $\times 10^9/L$	0.10 (0.04–0.24)	0.16 (0.01–0.50)	0.18 (0.02–1.16)†‡

Definition of abbreviations: reversibility FEV_1 = change in FEV_1 , expressed as increase in percentage predicted normal value after 400 mg of albuterol; ICS = inhaled corticosteroid; NO = nitric oxide; PC_{20} AMP = provocative concentration of adenosine 5' monophosphate causing a 20% fall in FEV_1 ; Phadiatop score = quotient specific IgE in patient serum/control serum; VC = inspiratory vital capacity.

Data are presented as median (range), unless stated otherwise. ICS dose is equivalent of beclomethasone. FEV_1 was determined after inhalation of 400 μ g of albuterol. Atopy is defined as specific IgE's in patient serum/control serum >1 . When applicable corrected for age, sex, and smoking using multiple regression analysis.

* $P < 0.05$; complete asthma remission versus clinical asthma remission.

† $P < 0.05$; complete asthma remission versus current asthma.

‡ $P < 0.05$; clinical asthma remission versus current asthma.

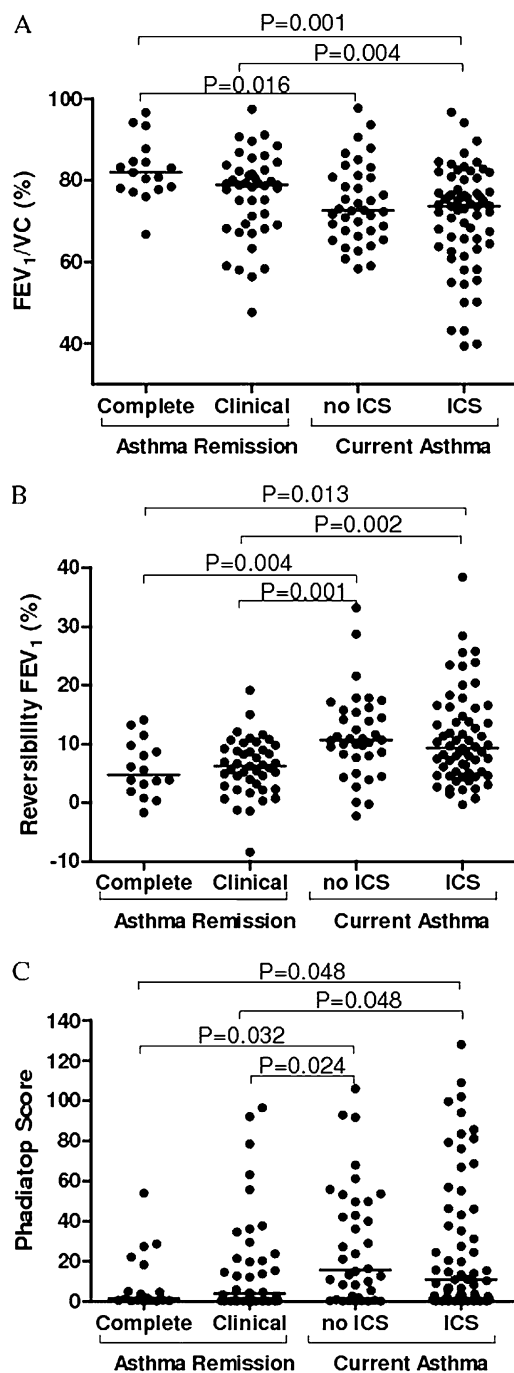


Figure 1. Patient characteristics. (A) FEV₁/VC (%), (B) reversibility of FEV₁ (% predicted), and (C) Phadiatop score in subjects with complete asthma remission, clinical asthma remission, and no inhaled corticosteroids (ICS) using patients with current asthma, and ICS using patients with current asthma. *P* values of FEV₁/VC are after correction for age, sex, and smoking using multiple regression analysis. Each circle represents one subject. Horizontal bars represent median values.

RESULTS

Patient Characteristics

In total, 165 former patients with asthma were investigated: 18 subjects with CoR, 44 with ClinR, and 103 CuA. Table 1 presents their clinical characteristics. Subjects with CoR and subjects in ClinR had a significantly higher FEV₁/VC (inspiratory vital capacity) compared with CuA (Table 1, Figure 1A).

Reversibility of FEV₁ was significantly lower in both CoR and ClinR compared with CuA (Table 1, Figure 1B). The Phadiatop score, a measure for the severity of atopy, was significantly lower in subjects with both CoR and ClinR compared with CuA (Table 1, Figure 1C). Alveolar and bronchial fractions of exhaled breath nitric oxide were not significantly different between the three groups (Table 1). Levels of alveolar and bronchial nitric oxide fractions correlated with blood eosinophil numbers in the complete population (CoR, ClinR, and CuA combined, $r_s = 0.39$, $P < 0.001$ and $r_s = 0.37$, $P < 0.001$, respectively).

Eosinophilic Inflammation in Blood, Sputum, and Biopsies

The number of blood eosinophils was significantly lower in both CoR and ClinR than in CuA (Table 1, Figure 2A).

The percentage of sputum eosinophils and the ECP concentration in sputum supernatant were not significantly different between the three groups (Table 2, Figures 2B and 2C). However, when the group with CuA was divided into non-ICS users and ICS users, sputum ECP was significantly higher in patients with CuA with ICS use than in those with CoR and CuA without ICS use (Figure 2C). Sputum ECP correlated significantly with the percentage of sputum eosinophils in the complete population (CoR, ClinR, and CuA combined, $r_s = 0.37$, $P < 0.001$).

EPX immunopositivity in the bronchial biopsy tissue was significantly lower in subjects with CoR than in subjects with ClinR and CuA (with and without ICS use) (Table 3, Figure 2D).

Eosinophilic inflammation in blood, sputum, and biopsy correlated significantly with each other in the complete population (CoR, ClinR, and CuA combined) (blood eosinophils vs. sputum eosinophils [%], $r_s = 0.54$, $P < 0.001$; blood eosinophils vs. EPX immunopositivity, $r_s = 0.25$, $P = 0.005$; and sputum eosinophils [%] vs. EPX immunopositivity, $r_s = 0.39$, $P < 0.001$).

Other Inflammatory Cells and Mediators in Sputum and Biopsies

The percentage of sputum lymphocytes was significantly higher in subjects with CoR than in CuA (Table 2). This was found in particular in CuA with ICS use (1.4 [0–7.3] % in CoR vs. 0.5 [0–6.9] % in CuA without ICS and 0.3 [0–4.1] % in CuA with ICS).

The level of histamine in sputum supernatant was significantly lower in subjects with ClinR compared with CuA (Table 2), and particularly lower than in CuA using ICS (18.5 [0–158.7] ng/ml in ClinR vs. 22.7 [0–139.9] ng/ml in CuA without ICS and 41.7 [4.7–215] ng/ml in CuA with ICS).

Besides group differences in eosinophils, no significant differences were observed in any of the other inflammatory cell types studied in bronchial biopsies (Table 3).

Airway Wall Remodeling

Basement membrane (BM) thickness was similar in CoR and ClinR. BM thickness was significantly increased in ClinR compared with CuA (Table 4, Figure 3). When ICS use was taken into account, BM thickness was significantly higher in both ClinR and CuA without ICS use than in CuA with ICS use (Figure 3).

No significant differences were observed between the three groups in any of the other investigated remodeling parameters (Table 4).

DISCUSSION

It has been long debated whether asthma can go into true remission. Our study shows that asthma can go into remission when considering the clinical characteristics of asthma (i.e.,

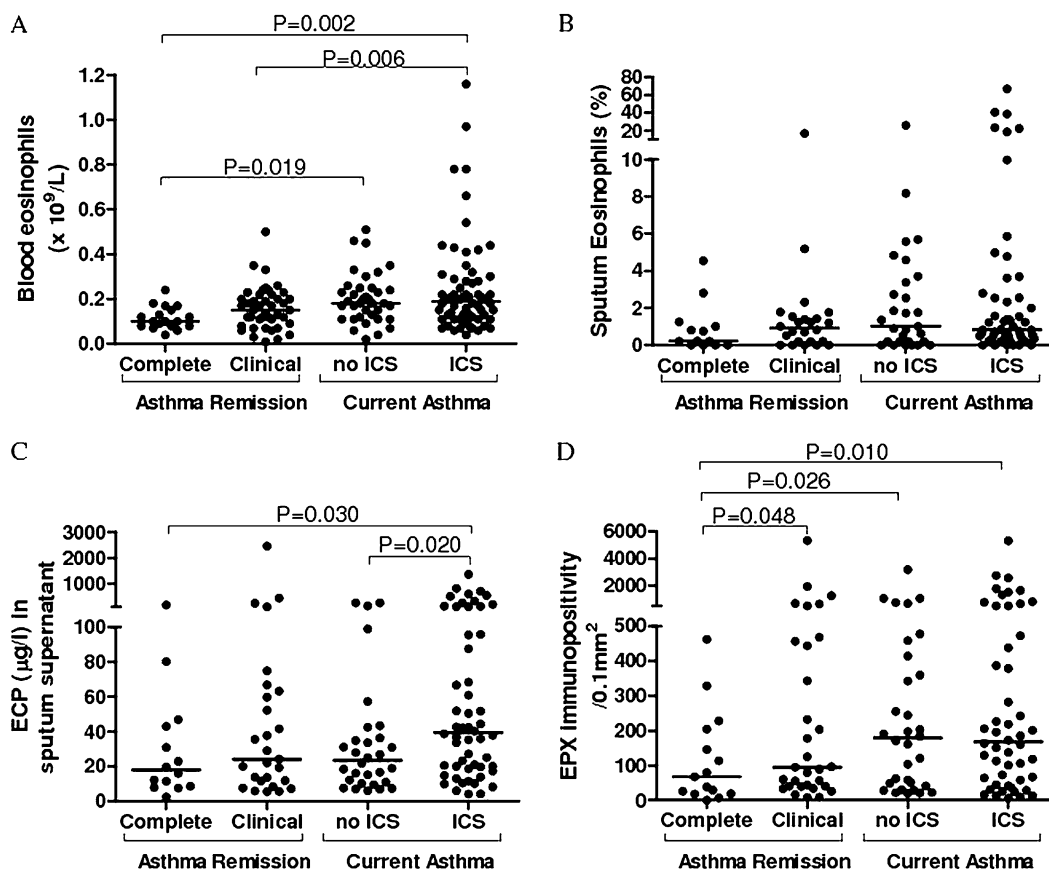


Figure 2. Eosinophilic inflammation. (A) Absolute blood eosinophils ($\times 10^9/L$), (B) percentage of sputum eosinophils, (C) eosinophil cationic protein (ECP) in sputum supernatant ($\mu g/L$), and (D) eosinophilic peroxidase (EPX) immunopositivity (pixels) in bronchial biopsies in subjects with complete asthma remission, clinical asthma remission, no inhaled corticosteroids (ICS) using patients with current asthma, and ICS using patients with current asthma. *P* values of EPX immunopositivity are after correction for age, sex, and smoking using multiple regression analysis. Each circle represents one subject. Horizontal bars represent median values.

those in remission had no asthma symptoms, had no BHR, and had normal lung function despite not using asthma treatment for at least 3 years). We demonstrate, however, that airway remodeling persists, as evidenced by a similar BM thickness in those with CoR, with ClinR, and in CuA without ICS use. Of particular interest is the observation that eosinophilic inflammation was best differentiating CoR from CuA. Moreover, eosinophilic activation in the airway wall, as indicated by increased degranulation in the EPX staining, was significantly lower in subjects with CoR than in those with ClinR and with CuA.

It has been previously shown that subjects with ClinR (no asthma symptoms and medication for 12 mo) had 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 BM when compared with control subjects without asthma (4). However, these individuals still had BHR and it is evident that persistence of BHR is abnormal. Also, asymptomatic BHR is a risk factor for future development of asthma symptoms in epidemiologic studies (9). Thus, our study supports our previous findings (6, 10) that it is indeed possible that patients with asthma who have expressed

TABLE 2. INFLAMMATORY CELLS AND MEDIATORS IN INDUCED SPUTUM

	Complete Asthma Remission (n = 18)	Clinical Asthma Remission (n = 44)	Current Asthma (n = 103)
Sputum not evaluable, n (%)	5 (28)	20 (45)	22 (21)
No sputum, n (%)	3 (16)	13 (29)	12 (11)
>80% squamous, n (%)	1 (6)	4 (9)	7 (7)
<600 cells, n (%)	1 (6)	3 (7)	3 (3)
Sputum evaluable, n (%)	13 (72)	24 (55)	81 (79)
Total cells, $\times 10^6/L$	0.31 (0.05–2.90)	0.44 (0.06–2.27)	0.43 (0.03–3.86)
Neutrophils, %	62 (19–78)	58 (21–94)	57 (16–94)
Macrophages, %	36 (20–78)	34 (5–77)	36 (5–83)
Eosinophils, %	0.2 (0–4.6)	0.9 (0–16.8)	0.9 (0–67.1)
Lymphocytes, %	1.4 (0–7.3)	0.7 (0–5.6)	0.4 (0–6.9)*
Neutrophil elastase, $\mu g/ml$	1.1 (0–5)	1.6 (0.2–5)	1.4 (0.2–5)
Eosinophilic cationic protein, $\mu g/L$	17.9 (2.5–172)	24.3 (5.6–2,467)	33.6 (4.2–1,363)
Histamine, ng/ml	22.7 (2.8–99.6)	18.5 (0–158.7)	35.4 (0–215)†

Data are presented as median (percentage or range). When applicable corrected for age, sex, and smoking using multiple regression analysis.

* $P < 0.05$, complete asthma remission versus current asthma.

† $P < 0.05$, clinical asthma remission versus current asthma.

TABLE 3. INFLAMMATORY CELLS IN BRONCHIAL BIOPSIES

	Complete Asthma Remission (n = 15)	Clinical Asthma Remission (n = 32)	Current Asthma (n = 82)
<i>Submucosal</i>			
CD3 ⁺ T-cells	52 (32–291)	67 (13–294)	68 (4–219)
CD4 ⁺ T-cells	12 (0–168)	21 (0–67)	17 (0–71)
CD8 ⁺ T-cells	28 (7–112)	18 (3–103)	23 (0–205)
CD20 ⁺ B-cells	2 (0–71)	4 (0–98)	3 (0–56)
NP57 ⁺ neutrophils	7 (2–30)	8 (0–46)	6 (0–38)
CD68 ⁺ macrophages	13 (2–86)	16 (0–37)	12 (0–57)
EPX ⁺ eosinophils	1 (0–10)	2 (0–40)	2 (0–32)
AA1 ⁺ mast cells	7 (1–26)	8 (0–20)	8 (0–26)
<i>(De)granulation</i>			
EPX immunopositivity, pixels	67 (0.5–462)	95 (8–5,329)*	172 (6–5,313)†
Degranulated AA1 ⁺ mast cells, %	71 (20–100)	80 (22–100)	68 (0–100)
<i>Smooth Muscle</i>			
AA1 ⁺ mast cells	1 (0–2)	1 (0–15)	1 (0–6)

Data are presented as median (range). The cell numbers are expressed per 0.1 mm² of tissue. When applicable corrected for age, sex, and smoking using multiple regression analysis. EPX = eosinophilic peroxidase.

* $P < 0.05$, complete asthma remission versus clinical asthma remission.

† $P < 0.05$, complete asthma remission versus current asthma.

BHR accompanied by asthma signs and symptoms are capable of losing these clinical hallmarks of asthma. We have previously published in a different group of patients with asthma that BM thickness was significantly larger in people with asthma than in age- and smoking matched healthy individuals (11). In a small sample of nine patients we previously found that BM thickness was similar in individuals with asthma remission; with fast decline in lung function (n = 11); and with slow decline in lung function (n = 18) (12). The current study extends this finding in a larger group (n = 18 with complete remission, 44 with clinical remission, and 103 with active asthma) and suggests that, even with CoR, some remodeling remains in the airways as in ongoing active asthma, likely as a sign of past disease or scarring.

There are some limitations to our study. We investigated individuals with asthma at middle age and remission may have different underlying pathology in childhood remission. It was not possible to assess the duration of asthma in our study population, because at initial testing, when all participants had active asthma, it was not asked at which age asthma started. Therefore, we cannot exclude that duration of asthma may have contributed to our findings. Nevertheless, we have investigated a considerable number of adults with asthma with CoR and our findings suggest that at this age remodeling may still be present. Finally, we found that the percentage of sputum lymphocytes was significantly higher in subjects with CoR than in CuA, particularly in CuA with ICS use. Because we did not perform

subset analysis of these lymphocytes further interpretation of this part of the results is not possible.

Individuals with asthma with remission, be it CoR or ClinR, were less frequently atopic and had significantly lower Phadia-top scores, implying less severe atopy than those with CuA. This was paralleled by differences in eosinophilic activation in airway wall biopsies and may well reflect that individuals with asthma who are in remission are less hampered by allergic stimuli as present in the environment. It remains to be established whether the lower atopic prevalence in those with remission is caused by loss of allergies during ageing, as has been shown previously (5). However, this will not likely affect results of our study because all groups had a comparable age of around 50 years when investigated. It is of interest that sputum eosinophil percentages were not significantly different in subjects with and without remission, suggesting that it is not merely the presence of eosinophils, but their activation state in the airway wall, as reflected by EPX staining, that drives their significant role in active disease. Sputum may thus constitute a waste basket of the presence of eosinophils in the airway wall, where it is the activation of these eosinophils that contributes to active asthma.

It was an unexpected finding that symptomatic patients with CuA using ICS had more severe eosinophilic inflammation than those not using ICS, as shown by higher blood eosinophil numbers and sputum ECP. This may reflect that asthma was

TABLE 4. REMODELING IN BRONCHIAL BIOPSIES

	Complete Asthma Remission (n = 15)	Clinical Asthma Remission (n = 32)	Current Asthma (n = 82)
<i>Bronchial epithelium</i>			
Goblet cells, cells/mm BM	34 (0–73)	32 (2–220)	36 (5–119)
Normal epithelium, %	6.8 (0–32.2)	8.9 (0–29.1)	9.0 (0–65.3)
Metaplasia, %	0 (0–21.2)	0 (0–26.2)	0 (0–19.4)
Ki67 ⁺ intact epithelium, %	2.9 (0–43.1)	2.8 (0–13.6)	3.7 (0–24.2)
Ki67 ⁺ basal epithelium, %	2.7 (0.2–19.2)	3.1 (0.1–15.5)	3.1 (0–28.9)
E-cadherin ⁺ intact epithelium, %	100 (0–100)	100 (0–100)	86.4 (0–100)
<i>Basement membrane</i>			
Thickness, μ m	6.3 (4.7–8.4)	6.5 (3.8–11.7)	5.7 (2.8–12.6)*
<i>Submucosa</i>			
Collagen 3, % stained tissue	38.8 (14.5–54.9)	36.7 (8.2–64.5)	41.9 (13.9–77.9)

Data are presented as median (range). When applicable corrected for age, sex, and smoking using multiple regression analysis.

* $P < 0.05$, clinical asthma remission versus current asthma.

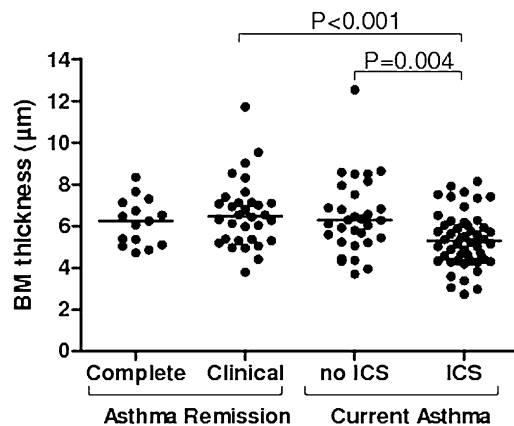


Figure 3. Airway remodeling. Basement membrane (BM) thickness in subjects with complete asthma remission, clinical asthma remission, no inhaled corticosteroids (ICS) using patients with current asthma, and ICS using patients with current asthma. *P* values are after correction for age, sex, and smoking using multiple regression analysis. Each circle represents one subject. Horizontal bars represent median values.

not under control in patients with CuA using ICS, and higher doses may be necessary to reach control (13). As already mentioned, BM thickness was lower in patients with CuA using ICS, showing that asthma symptoms are not driven by this remodeling feature.

Functional abnormalities in asthma, such as BHR, are thought to be caused by airway inflammation or structural changes of the airways, such as smooth muscle hyperplasia, airway wall thickening, and edema. These structural changes, known as “airway remodeling,” are thought to appear early in the onset of the disease, sometimes even before symptoms appear (14). The exact physiologic consequences of airway wall remodeling are not understood. We show here that airway remodeling is also present in subjects in CoR. This suggests that BM thickening *per se* is not responsible for the presence of BHR. In contrast, activation of eosinophils in the airway wall is almost absent in CoR and present in those with CuA, suggesting that this may contribute to the presence or severity of BHR. Nevertheless, other factors also have to drive the presence and severity of BHR, because eosinophilia was absent in a proportion of our patients with CuA.

The question arises if airway remodeling is an inherent feature of asthma (i.e., if it remains even though at extensive clinical examination asthma is in CoR). If this is true, then one might interpret the presence of increased BM thickening as a pending risk of asthma relapse. Alternatively, this merely reflects an end-stage disease with some “scarring” as a sign that an individual has had asthma. There are indications that remodeling is a process that is amenable to treatment. Indeed, two studies have shown that prolonged treatment with ICS can reduce BM thickness (15, 16). Moreover, collagen deposition (17) and airway vascularity (18) can improve with ICS use and are therefore also reversible. In light of these observations it is of interest that patients with CuA using ICS in our study had lower BM thickness than those without ICS. This may thus simply result from ICS treatment for a prolonged time. However, despite this, patients still remain symptomatic. Therefore, we conclude that BM thickness itself does not associate with the clinical expression of asthma, but that other factors imprint on BM thickness that affect the severity of the disease and treatment requirements.

Epithelial cells are the first barrier against inhaled allergens; hence, these cells may be important for the induction, maintenance, and severity of asthma. We have previously shown that

individuals with asthma who smoke have more respiratory symptoms and epithelial damage than individuals with asthma who do not smoke (7). We had anticipated that patients with CuA would have more epithelial changes than those with ClinR and CoR. However, the percentage of intact epithelium and its expression of E-cadherin, an adhesion protein, were similar between the groups. With respect to epithelial damage, we conclude that this does not differentiate remission from CuA. This does, of course, not preclude that the activation status of epithelial cells is different. It may well be that the production of chemoattractive cytokines by epithelium is increased in CuA compared with remission, which then adds to attracting eosinophils to the airway wall as observed in our study.

In summary, we studied a group of 164 adults with known active asthma in the past, including individuals with CoR (i.e., those being asymptomatic without airway obstruction, BHR, and any respiratory treatment), with ClinR (i.e., asymptomatic and without treatment); and with CuA. The important message of this paper is that asthma can go into full remission according to all clinical phenotypes that characterize asthma, but that BM thickening, which accompanies asthma, persists. A second message of the paper is that airway wall eosinophilic activation differentiates CoR from ClinR and CuA. This signifies the importance of eosinophil activation for the clinical expression of asthma and the severity of BHR.

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References

- Holm M, Omenaas E, Gislason T, Svanes C, Jogi R, Norrman E, Janson C, Toren K. Remission of asthma: a prospective longitudinal study from northern Europe (RHINE study). *Eur Respir J* 2007;30:62–65.
- Boulet LP, Turcotte H, Brochu A. Persistence of airway obstruction and hyperresponsiveness in subjects with asthma remission. *Chest* 1994; 105:1024–1031.
- Warke TJ, Fitch PS, Brown V, Taylor R, Lyons JD, Ennis M, Shields MD. Outgrown asthma does not mean no airways inflammation. *Eur Respir J* 2002;19:284–287.
- van den Toorn LM, Overbeek SE, de Jongste JC, Leman K, Hoogsteden HC, Prins JB. Airway inflammation is present during clinical remission of atopic asthma. *Am J Respir Crit Care Med* 2001;164:2107–2113.
- Panhuysen CI, Vonk JM, Koeter GH, Schouten JP, van Altena R, Bleecker ER, Postma DS. Adult patients may outgrow their asthma: a 25-year follow-up study. *Am J Respir Crit Care Med* 1997;155:1267–1272.
- Vonk JM, Postma DS, Boezen HM, Grol MH, Schouten JP, Koeter GH, Gerritsen J. Childhood factors associated with asthma remission after 30 year follow up. *Thorax* 2004;59:925–929.
- Broekema M, Ten Hacken NH, Volbeda F, Lodewijk ME, Hylkema MN, Postma DS, Timens W. Airway epithelial changes in smoking but not in ex-smoking asthmatics. *Am J Respir Crit Care Med* 2009; 180:1170–1178.
- Protheroe C, Woodruff SA, de Petris G, Mukkada V, Ochkur SI, Janarthanan S, Lewis JC, Pasha S, Lunsford T, Harris L, et al. A novel histologic scoring system to evaluate mucosal biopsies from patients with eosinophilic esophagitis. *Clin Gastroenterol Hepatol* 2009;7:749–755.

9. Peat JK, Toelle BG, Salome CM, Woolcock AJ. Predictive nature of bronchial responsiveness and respiratory symptoms in a one-year cohort study of Sydney schoolchildren. *Eur Respir J* 1993;6: 662–669.
10. Grol MH, Gerritsen J, Vonk JM, Schouten JP, Koeter GH, Rijcken B, Postma DS. Risk factors for growth and decline of lung function in asthmatic individuals up to age 42 years. A 30-year follow-up study. *Am J Respir Crit Care Med* 1999;160:1830–1837.
11. Liesker JJ, Ten Hacken NH, Zeinstra-Smith M, Rutgers SR, Postma DS, Timens W. Reticular basement membrane in asthma and COPD: similar thickness, yet different composition. *Int J Chron Obstruct Pulmon Dis* 2009;4:127–135.
12. Broekema M, Volbeda F, Timens W, Dijkstra A, Lee NA, Lee JJ, Lodewijk ME, Postma DS, Hylkema MN, Ten Hacken NHT. Airway eosinophilia in remission and progression of asthma: accumulation with a fast decline of FEV1. *Respir Med* 2010;104:1–9.
13. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, Gibson P, Ohta K, O'Byrne P, Pedersen SE, *et al.* Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008;31:143–178.
14. Cokugras H, Akcakaya N, Seckin I, Camcioglu Y, Sarimurat N, Aksoy F. Ultrastructural examination of bronchial biopsy specimens from children with moderate asthma. *Thorax* 2001;56:25–29.
15. 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;159:1043–1051.
16. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, Walters EH. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax* 2002;57:309–316.
17. Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999;104:356–363.
18. Orsida BE, Ward C, Li X, Bish R, Wilson JW, Thien F, Walters EH. Effect of a long-acting beta(2)-agonist over three months on airway wall vascular remodeling in asthma. *Am J Respir Crit Care Med* 2001; 164:117–121.

Persisting remodeling and less airway wall eosinophil activation in complete remission of asthma

Martine Broekema, Wim Timens, Judith M. Vonk, Franke Volbeda, Monique E. Lodewijk, Machteld N. Hylkema, Nick H.T. ten Hacken and Dirkje S. Postma

Online data supplement

Exclusion criteria

Main exclusion criteria were: FEV₁ <1.2 L, bronchiectasis, upper respiratory tract infection (e.g. colds) and/or use of antibiotics or oral corticosteroids within the last 2 months before inclusion.

Lung function

Lung function tests were performed with a daily calibrated spirometer according to standardized guidelines as described previously^{E1}. FEV₁ and VC (inspiratory vital capacity) was measured with a calibrated water-sealed spirometer according to standardized guidelines^{E1;2}. Reversibility of FEV₁ (% predicted) was measured after administration of 400µg Albuterol. BHR to AMP was tested with a method adapted from Cockcroft and coworkers^{E3} using, after an initial nebulized 0.9% saline challenge, 2-min tidal breathing at 5-min intervals and doubling concentrations of AMP ranging from 0.04 to 320 mg/ml. BHR to histamine was tested as reported previously^{E4}, using 30-second tidal breathing and doubling concentrations of histamine ranging from 0.13 to 32 mg/ml.

Alveolar and bronchial nitric oxide

Exhaled nitric oxide (NO) was measured on the Aerocrine NO system (Niox, Aerocrine AB, Stockholm, Sweden) in accordance with international guidelines^{E5}. Alveolar NO concentrations (ppb) and bronchial NO fluxes (nL/s) were assessed according to Tsoukias and George^{E6} with some modifications^{E7}.

Atopy

Atopy was determined by the Phadiatop screening test^{E8} with the ImmunoCap system (Phadia AB, Uppsala, Sweden). The Phadiatop score is expressed as quotient, i.e. fluorescence of the serum of interest divided by the fluorescence of a control serum. Positive Phadiatop, and thereby atopy, was defined as serum/control serum >1.

Induced sputum

Sputum was induced by 5% hypertonic saline aerosols over 3 consecutive periods of 5 min. Cytospins of processed whole sputum samples were stained with May-Grünwald-Giemsa and cell differentials were determined by a blinded observer by counting a total of 600 viable, non-squamous cells. Sputum was not used if percentage of squamous cells was greater than 80% or when the total number of non-squamous cells exceeded 600. Histamine (IBL, Hamburg, Germany) and neutrophil elastase (Hycult, Uden, the Netherlands) in sputum supernatant were determined by ELISA and eosinophilic cationic protein (ECP) by a fluoroenzyme immunoassay (ImmunoCAP ECP, Phadia AB).

Bronchial biopsies

After local anaesthesia, bronchial biopsies were obtained using a flexible bronchoscope (Olympus BF P20 or BF XT20) from segmental divisions of the main bronchi. Biopsies were fixed in 4% formalin, processed and embedded in paraffin and cut in 3µm thick sections. Morphological features were determined on sections stained with hematoxylin and eosin (HE) and with periodic acid Schiff. Immunohistochemical stainings (except for Ki67 and E-cadherin) were performed using the DAKO autostainer (DAKO, Glostrup, Denmark). The slides were included in

a random fashion in each run to avoid groupwise staining. Immunohistochemistry was performed using antibodies directed against T-cells: (CD3, DAKO; CD4, Novocastra, Newcastle, UK; and CD8, DAKO), B-cells (CD20, DAKO), neutrophil elastase (NP57, DAKO), macrophages (CD68, DAKO), eosinophilic peroxidase (EPX^{E9}), mast cell tryptase (AA1, DAKO), proliferation (Ki67, DAKO), epithelial adhesion (E-cadherin, BD Biosciences, Breda, the Netherlands) and collagen 3 (Southern Biotech, Birmingham, AL, USA). In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) and the chromogen NovaRED (Vector Labs, Burlingame, USA). EPX was detected via biotinylated anti-mouse IgG1 (Southern Biotech), alkaline phosphatase-labeled conjugate (DAKO) and permanent Red (DAKO). Ki67, E-cadherin and collagen 3 were detected with two peroxidase-labelled conjugates (both DAKO) and 3,3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands).

All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). Quantification was performed on the largest of three biopsy sections. Inflammatory cell numbers were quantified by counting the number of positively stained cells in the submucosal area 100µm under the basement membrane (BM), in a total area of 0.1mm² per biopsy sample. Activation of eosinophils and mast cells was determined by their degree of degranulation. Degranulation of eosinophils was determined by computer-assisted quantification of EPX immunopositivity in the submucosa. The percentage of degranulated AA1⁺ cells was determined by counting intact (dense, compact AA1⁺ cells with unbroken boundaries) and degranulated mast cells (all other AA1⁺ cells), as described

previously^{E10}. Goblet cell numbers were counted on PAS-stained biopsy sections and expressed per mm of BM. Epithelial layer integrity was assessed on HE-stained biopsy sections and expressed as the percentage of BM covered with 1) normal, intact epithelium (basal and ciliated columnar epithelial cells) and 2) metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells). Epithelial proliferation was determined by counting the number of Ki67⁺ cells in intact epithelium and basal epithelium. Epithelial adhesion was determined by assessment of the percentage of BM covered with E-cadherin⁺ intact epithelium. BM thickness was calculated based on computer-assisted measurements of BM surface area and BM length. Submucosal deposition of collagen 3 was determined by computer-assisted image analysis in an area of 200 μ m under the BM.

Reference List

- E1. Meijer, R. J., H. A. Kerstjens, L. R. Arends, H. F. Kauffman, G. H. Koeter, and D. S. Postma. 1999. Effects of inhaled fluticasone and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 54:894-899.
- E2. Quanjer, P. H., G. J. Tammeling, J. E. Cotes, O. F. Pedersen, R. Peslin, and J. C. Yernault. 1994. [Lung volumes and forced ventilatory flows. Work Group on Standardization of Respiratory Function Tests. European Community for Coal and Steel. Official position of the European Respiratory Society]. *Rev.Mal Respir.* 11 Suppl 3:5-40.
- E3. Cockcroft, D. W., D. N. Killian, J. J. Mellon, and F. E. Hargreave. 1977. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin.Allergy* 7:235-243.
- E4. Grol, M. H., J. Gerritsen, J. M. Vonk, J. P. Schouten, G. H. Koeter, B. Rijcken, and D. S. Postma. 1999. Risk factors for growth and decline of lung function in asthmatic individuals up to age 42 years. A 30-year follow-up study. *Am.J.Respir.Crit Care Med.* 160:1830-1837.
- E5. 2005. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am.J.Respir.Crit Care Med.* 171:912-930.
- E6. Tsoukias, N. M. and S. C. George. 1998. A two-compartment model of pulmonary nitric oxide exchange dynamics. *J.Appl.Physiol* 85:653-666.

- E7. Rottier, B. L., J. Cohen, T. W. van der Mark, W. R. Douma, E. J. Duiverman, and N. H. ten Hacken. 2005. A different analysis applied to a mathematical model on output of exhaled nitric oxide. *J.Appl.Physiol* 99:378-379.
- E8. Eriksson, N. E. 1990. Allergy screening with Phadiatop and CAP Phadiatop in combination with a questionnaire in adults with asthma and rhinitis. *Allergy* 45:285-292.
- E9. Protheroe, C., S. A. Woodruff, G. de Petris, V. Mukkada, S. I. Ochkur, S. Janarthanan, J. C. Lewis, S. Pasha, T. Lunsford, L. Harris, et al. 2009. A novel histologic scoring system to evaluate mucosal biopsies from patients with eosinophilic esophagitis. *Clin.Gastroenterol.Hepatol.* 7:749-755.
- E10. Carroll, N. G., S. Mutavdzic, and A. L. James. 2002. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. *Eur.Respir.J.* 19:879-885.