

Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction

Marta Kaminska, MD,^a Susan Foley, MD,^a Karim Maghni, DSc, PhD,^b Claudine Storness-Bliss, BSc,^c Harvey Coxson, PhD,^c Heberto Ghezzi, PhD,^a Catherine Lemi re, MD, MSc,^b Ronald Olivenstein, MD,^a Pierre Ernst, MD,^a Qutayba Hamid, MD, PhD,^a and James Martin, MD, DSc^a Montreal, Quebec, and Vancouver, British Columbia, Canada

Background: The patterns of airway remodeling and the biomarkers that distinguish different subtypes of severe asthma are unknown.

Objectives: We sought to characterize subjects with severe asthma with and without chronic persistent airflow obstruction with respect to airway wall remodeling (histopathologic and radiologic) and specific sputum biomarkers.

Methods: Subjects with severe asthma with chronic persistent (n = 16) or intermittent (n = 18) obstruction were studied. Endobronchial biopsy specimens were analyzed for airway smooth muscle area, epithelial detachment, basement membrane thickness, and submucosal fibrosis. Levels of eosinophil cationic protein, myeloperoxidase, matrix metalloproteinase 9, tissue inhibitor of matrix metalloproteinase 1 (ELISA), and 27 cytokines (multiplex assay) and differential cell counts were measured in induced sputum. Airway thickness was measured by means of high-resolution computed tomographic scanning.

Results: Chronic persistent obstruction was associated with earlier age of onset, longer disease duration, more inflammatory cells in the sputum, and greater smooth muscle area ($15.65\% \pm 2.69\%$ [n = 10] vs $8.96\% \pm 1.99\%$ [n = 14], $P = .0325$). No differences between groups were found for any of the biomarker molecules measured in sputum individually. However, principal component analysis revealed that the dominant variables in the chronic persistent obstruction group were IL-12, IL-13, and IFN- γ , whereas IL-9, IL-17, monocyte chemoattractant protein 1,

and RANTES were dominant in the other group. Airway imaging revealed no differences between groups.

Conclusion: Subjects with severe asthma with chronic persistent obstruction have increased airway smooth muscle with ongoing T_H1 and T_H2 inflammatory responses. Neither airway measurements on high-resolution computed tomographic scans nor sputum analysis seem able to identify such patients.

(J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Severe asthma, remodeling, airway smooth muscle, fibrosis, reticular basement membrane, inflammation, cytokines, high-resolution computed tomographic scan, biopsy, sputum

Severe asthma, although it accounts for only 5% to 10% of asthma cases, leads to a disproportionate amount of health care resource use.¹ Treatment is challenging, requiring continual oral or high-dose inhaled corticosteroids, with variable treatment success.² Severe asthma is heterogeneous, with lung function that is seemingly irreversibly reduced in some but intermittently normal in others. It has been suggested that progressive remodeling might lead to irreversibility of airway dysfunction.³ Some features of remodeling, particularly increased airway smooth muscle (ASM), have been associated with asthma severity.⁴⁻⁶ However, the basis for irreversible airway obstruction is currently not known, and whether it is associated with any distinct pattern of remodeling has not been elucidated.

Our hypothesis was that chronic persistent airflow obstruction is associated with a greater degree of airway remodeling compared with that seen in subjects with severe asthma without this pattern of obstruction. Specifically, primary outcomes were differences in airway wall fibrosis and ASM between groups. Reticular basement membrane (RBM) thickness and epithelial detachment were also examined. Clinical and radiographic characteristics that might be predictive of chronic persistent obstruction were assessed. In addition, an exploratory characterization of sputum biomarkers was done based on the reasoning that the pattern of airway remodeling might be related to a distinct pattern of inflammation that is measurable in a noninvasive manner through the analysis of induced sputum.⁷ Subjects were followed over a 13-month period and underwent bronchoscopic biopsy of the proximal airways for assessment of remodeling and high-resolution computed tomographic (HRCT) scanning.

METHODS

Asthmatic subjects

Subjects were recruited at the McGill University Health Centre and at the H pital du Sacre-C ur of the Universit  de Montr al, Montreal, Canada. The

From ^aMeakins-Christie Laboratories, Montreal; ^bCentre de Recherche de l'H pital du Sacre-C ur de Montr al, Montreal; and ^cthe Department of Radiology and the James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, Vancouver General Hospital.

Supported by the Richard and Edith Strauss Canada Foundation, Canadian Institutes of Health Research.

Disclosure of potential conflict of interest: H. Coxson has served as a consultant/advisor board member for GlaxoSmithKline and has received research support from GlaxoSmithKline, Spiration, and Wyeth. C. Lemi re has received research support from the National Institute for Occupational Safety and Health and the Institut de Recherche Robert-Sauv  en Sant  et S curit  du Travail and has served as an advisor to GlaxoSmithKline, AstraZeneca, and Novartis. P. Ernst has received speaker's fees from or served on an advisory board for AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck, Novartis, and Nycomed. Q. Hamid has received research support from the McGill University Health Center Strauss Foundation. The rest of the authors have declared that they have no conflict of interest.

Received for publication July 31, 2008; revised March 4, 2009; accepted for publication March 16, 2009.

Reprint requests: James Martin, MD, DSc, Meakins Christie Laboratories, 3626 St Urbain, Montreal, Quebec, Canada, H2X 2P2. E-mail: james.martin@mcgill.ca.
0091-6749/\$36.00

  2009 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2009.03.049

Abbreviations used

ASM:	Airway smooth muscle
ATS:	American Thoracic Society
CT:	Computed tomography
ECP:	Eosinophil cationic protein
FeNO:	Fraction of exhaled nitric oxide
HRCT:	High-resolution computed tomography
MCP:	Monocyte chemotactic protein
MIP:	Macrophage inflammatory protein
MMP:	Matrix metalloproteinase
MPO:	Myeloperoxidase
RBM:	Reticular basement membrane
SMA:	Smooth muscle area
TIMP:	Tissue inhibitor of matrix metalloproteinase
WA%:	Wall area percentage

protocols were reviewed and approved by the ethics review board of each institution. Written informed consent was obtained. All subjects were adult nonsmokers or exsmokers for at least 2 years and with a smoking history of less than 15 pack-years. Severe asthma was defined in accordance with the American Thoracic Society (ATS) workshop on refractory asthma.⁸ Because we were interested in studying only asthmatic subjects with truly severe disease despite the best available treatment and not undertreated subjects, only those found to be compliant with the prescribed combination inhaler of fluticasone and salmeterol during a 1-month trial period were included. All continued to use this combination metered-dose inhaler treatment throughout the study (Table I). See the **Methods** section in this article's Online Repository at www.jacionline.org for details and exclusion criteria. Subjects were seen monthly for 13 months. Chronic persistent airflow obstruction was defined as an FEV₁ of less than 70% of predicted value at each visit. One value above this cutoff point but less than 75% of predicted value was accepted. All other subjects were considered to be able to achieve a normal or near-normal FEV₁ and were assigned to the group with intermittent obstruction.

Procedures

Study procedures were described previously (see the **Methods** section in this article's Online Repository for details).⁹ In brief, sputum was induced by using inhalation of increasing concentrations (3%, 4%, and 5%) of hypertonic saline and processed as previously described.¹⁰ The fraction of exhaled nitric oxide (FeNO) was measured with an offline chemiluminescence technique from sample bags in accordance with ATS recommendations.¹¹ Sputum and FeNO values from the visit immediately preceding the bronchoscopy were used in this analysis. During bronchoscopy, 6 endobronchial biopsies were done at various segmental and subsegmental carinae of the right lung. Tissues were fixed in formaldehyde and embedded in paraffin in random orientation before cutting. All slides were screened for adequacy of the section before staining in a blinded fashion as to the source subject. Staining was performed by using a peroxidase-based method. ASM was detected with the monoclonal anti- α smooth muscle actin antibody clone 1A4 (Sigma, St Louis, Mo) by using the DAB method (DakoCytomation, Inc, Glostrup, Denmark). Van Gieson stain was used to detect collagen fibers (fibrosis). RBM thickness and epithelial detachment were measured on hematoxylin and eosin-stained slides. Sections without mucosa and those containing predominantly cartilage were rejected. Because tissue from some subjects had been processed previously for other published studies,^{4,9,12} not all subjects had sufficient tissue available for all analyses.

Morphometry

Image analysis was performed with the Image Pro-Plus 4.0 system (Media Cybernetics, Silver Springs, Md). Images were recorded on a CCD color video camera (Sony, Montvale, NJ) mounted on a conventional light microscope (Olympus Optical Co, Tokyo, Japan). Smooth muscle area (SMA) was measured from images taken at a magnification of $\times 40$ on the microscope. A percentage of total biopsy area was calculated (SMA%). RBM thickness was

measured at a magnification of $\times 400X$, as proposed by Sullivan et al.¹³ Submucosal fibrosis was quantified on a scale of 1 to 4 according to the method of Minshall et al.¹⁴ The length of epithelial detachment was measured as a percentage of total epithelial length. Measurements were done in duplicate by 1 blinded observer.

Sputum supernatants

Sputum supernatants were kept frozen at -80°C until processing. After thawing, they were concentrated by means of centrifugation with Centricon centrifugal filters (YM-3; Millipore, Montreal, Quebec, Canada) with a 3,000-d molecular weight cutoff, as previously described.¹⁵ Eosinophil cationic protein (ECP; MBL Co, Ltd, Nagoya, Japan), myeloperoxidase (MPO; Immunology Consultants Laboratory, Inc, Newberg, Ore), matrix metalloproteinase (MMP) 9 (Raybiotech, Inc, Norcross, Ga), and tissue inhibitor of matrix metalloproteinase (TIMP) 1 (EMD-Calbiochem, San Diego, Calif) levels in concentrated sputum samples were measured by means of ELISA. Optical densities were determined with the ELISA reader Elx 808iu (Bio-Tek Instruments, Inc, Richmond, Va), and calculations were performed with KC4 software (Bio-Tek Instruments, Inc). Data were adjusted for the factor of concentration and expressed in picograms per milliliter.¹⁵ IL-1 β , IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor, granulocyte colony-stimulating factor, GM-CSF, IFN- γ , IFN- γ -induced protein 10, monocyte chemotactic protein (MCP) 1, macrophage inflammatory protein (MIP) α , MIP- β , platelet-derived growth factor bb, RANTES, TNF- α , and vascular endothelial growth factor were quantified in concentrated sputum samples by using a 27-plex assay (Bio-Rad, Mississauga, Ontario, Canada) and the Bio-Plex workstation based on the Luminex technology.

HRCT scans

All HRCT scans were acquired at suspended full inspiration. Airway wall analysis was done with the full-width-at-half-maximum principle (see the **Methods** section in this article's Online Repository for further details).

Analysis

Data were analyzed with the Wilcoxon rank sum test. Clinical data are shown as means $\pm$ SDs, and remodeling and biomarker data are shown as means $\pm$ SEMs. Raw principal component analysis was done for sputum supernatant cytokines by using a singular value decomposition (centered and scaled) data matrix with the software "R."¹⁶ Pearson correlation coefficients were calculated with SPSS for Windows, version 15.0 (SPSS, Inc, Chicago, Ill). A *P* value of less than .05 was considered statistically significant.

RESULTS**Clinical data**

Subject characteristics are shown in Table I. There was no difference between the subjects with and without chronic persistent obstruction in age, smoking history, Asthma Control Questionnaire score, or sputum differential cell count. However, the group with chronic persistent obstruction had a greater proportion of sputum neutrophils and eosinophils combined. This group also had an earlier age of onset and longer disease duration, as well as higher daily doses of long-acting bronchodilator and inhaled corticosteroid.

Airway remodeling

Features of airway remodeling were assessed on endobronchial biopsy specimens: ASM (area as a percentage of total biopsy area), submucosal fibrosis (score, 0-4), RBM thickness, and epithelial detachment. There was no difference in the mean total biopsy area between the 2 groups (chronic persistent obstruction, $1.161 \pm 0.552 \text{ mm}^2$ [SD]; intermittent obstruction, $1.002 \pm 0.301 \text{ mm}^2$ [SD]; *P* = .90). The SMA% was significantly greater in the group with chronic persistent obstruction than in the group

TABLE I. Clinical data

	Chronic persistent obstruction (FEV ₁ <70%)	Intermittent obstruction (FEV ₁ ≥70%)	P value
No.	16	18	
Age (y)	49.7 ± 10.6	45.0 ± 10.8	.39
Sex (F/M)	6/10	12/6	
Exsmokers, no. (pack-years)	4 (4.2 ± 7.5)	9 (4.9 ± 8.0)	.80
Atopy, no.	13	13	.63
Age of onset (y)	19.1 ± 18.1	32.9 ± 16.6	.007*
Disease duration (y)	30.4 ± 16.3	12.1 ± 10.2	.0009*
Asthma Control Questionnaire score	2.23 ± 1.00	2.12 ± 1.24	.83
Best FEV ₁ (% predicted)	56.1 ± 12.6	90.2 ± 14.3	<.00001*
FEV ₁ /FVC (%)	55.8 ± 11.5	70.9 ± 14.3	.005*
FeNO (ppb)	27.8 ± 23.0	18.5 ± 13.1	.27
Sputum eosinophils (%)	8.3 ± 17.9	7.9 ± 14.0	.11
Sputum neutrophils (%)	65.3 ± 30.1	48.9 ± 29.6	.07
Sputum eosinophils and neutrophils combined (%)	74.6 ± 22.9	52.6 ± 30.7	.03*
Daily ICS (μg)	1,331.4 ± 366.0	1,061.1 ± 314.7	.04*
Daily LABA (μg)	150.0 ± 50.0	108.3 ± 25.0	.006*
Daily oral corticosteroid, no. (mg/d)	4 (18.0 ± 9.08)	8 (12.1 ± 6.36)	.42

Values are presented as means ± SDs unless otherwise stated.

F, Female; M, male; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long-acting β-agonist.

*Statistically significant difference (Wilcoxon rank sum test).

with intermittent obstruction ($15.65\% \pm 2.69\%$ [$n = 10$] vs $8.96\% \pm 1.99\%$ [$n = 14$], $P = .033$; Fig 1). SMA% negatively correlated with FEV₁ (percent predicted) in all subjects (Fig 2), with a trend in the chronic persistent obstruction group ($r = -0.594$, $P = .07$) but not in the intermittent obstruction group ($r = -0.215$, $P =$ not significant), possibly because of the ceiling effect in FEV₁ values. SMA% was assessed in relation to smoking history to address potential confounding by prior smoking history. No difference was found between exsmokers and never smokers, nor was there any significant correlation between number of pack-years smoked and SMA% (data not shown). Submucosal fibrosis showed a trend toward an increase in subjects with chronic persistent obstruction (chronic persistent obstruction, 2.87 ± 0.245 [$n = 8$]; intermittent obstruction, 2.27 ± 0.237 [$n = 11$]; $P = .07$), although this was not statistically significant. There was no difference between the 2 groups in RBM thickness (chronic persistent obstruction, 4.57 ± 0.465 μm [$n = 9$]; intermittent obstruction, 4.67 ± 0.374 μm [$n = 14$]; $P = .43$) or epithelial detachment (chronic persistent obstruction, $27.7\% \pm 9.48\%$ [$n = 9$]; intermittent obstruction, $21.1\% \pm 3.23\%$ [$n = 11$]; $P = .44$).

Markers of granulocyte activation

Levels of ECP and MPO, markers of eosinophil and neutrophil activation respectively, were measured in sputum supernatants. There was no correlation between these markers and the corresponding differential cell counts. There was no significant difference between the groups with chronic persistent obstruction ($n = 14$) and intermittent obstruction ($n = 17$) in either sputum ECP (23.8 ± 30.7 vs 40.5 ± 93.0 ng/mL, respectively; $P = .95$) or MPO (99.4 ± 107.4 vs 196.7 ± 30.2 ng/mL, respectively; $P = 1.0$) levels. There was, however, a positive correlation between ECP and SMA% on biopsy in the 2 groups combined ($r = 0.534$, $P = .05$ [$n = 20$]).

MMP-9 and TIMP-1

MMP-9 and TIMP-1 levels were measured in concentrated sputum samples. No correlations were found with sputum

differential cell counts. No significant differences between the groups with chronic obstruction ($n = 14$) and intermittent obstruction ($n = 17$) were noted in either MMP-9 (790.8 ± 1026.9 vs 1582.29 ± 2090.0 ng/mL, respectively; $P = .58$) or TIMP-1 (55.4 ± 100.5 vs 33.1 ± 52.8 ng/mL, respectively; $P = .68$) levels or their molar ratio (MMP-9/TIMP-1; 17.4 ± 20.7 vs 23.7 ± 35.1 , respectively; $P = .15$). MMP-9 gelatinase activity in sputum samples was confirmed by means of zymography (data not shown).

Cytokine and chemokine profile

A multiplex assay was used to quantify the following cytokines and chemokines in concentrated sputum samples from 9 subjects with and 14 subjects without chronic persistent obstruction: IL-1β, IL-1RA, IL-2, IL-4 to IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor, granulocyte colony-stimulating factor, GM-CSF, IFN-γ, IFN-γ-induced protein 10, MCP-1, MIP-α, MIP-β, platelet-derived growth factor bb, RANTES, TNF-α, and vascular endothelial growth factor (see Table E1 in this article's Online Repository at www.jacionline.org for further details). No differences were found between the groups. However, correlations were found for all subjects between SMA% and the following cytokines: IL-5 ($r = 0.623$, $P = .01$), IL-12 ($r = 0.462$, $P = .05$), IFN-γ ($r = 0.507$, $P = .05$), and IL-13 ($r = 0.551$, $P = .01$; see Fig E1 in this article's Online Repository at www.jacionline.org). When analyses were restricted to values above detection limits, the following correlations emerged: IL-5 ($r = 0.94$, $P = .006$, $n = 6$); IL-12 ($r = 0.82$, $P = .001$, $n = 12$); rIFN-γ ($r = 0.71$, $P = .021$, $n = 10$); and IL-13 ($r = 0.74$, $P = .058$, $n = 13$). Principal component analysis revealed that the dominant variables were IL-12, IL-13, and IFN-γ in the group with chronic obstruction and IL-9, IL-17, MCP-1, and RANTES in the group with intermittent obstruction.

Assessment of remodeling by means of HRCT

We assessed whether airway imaging by means of HRCT scanning could detect remodeling associated with chronic

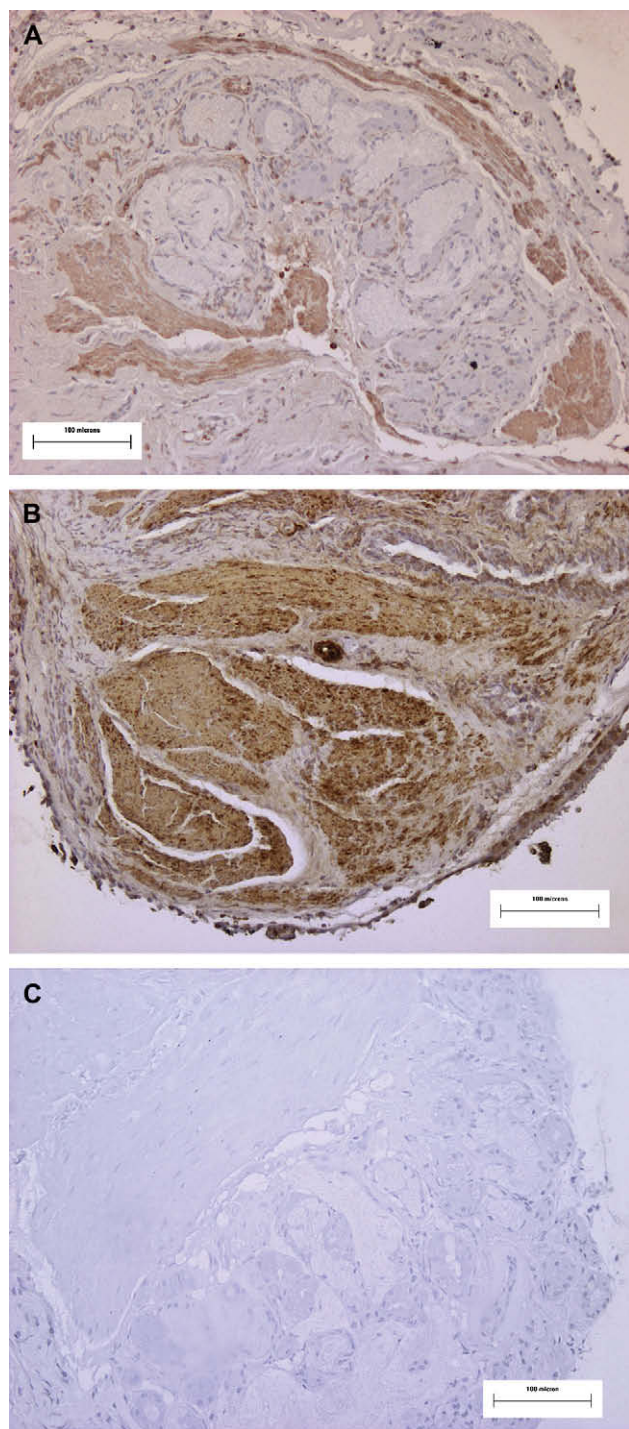


FIG 1. ASM in endobronchial biopsy specimens. Smooth muscle bundles (shown in brown) are larger in a subject with chronic persistent obstruction (**A**) than in a subject with intermittent obstruction (**B**). **C**, Isotype control (α -smooth muscle actin immunoperoxidase staining).

persistent obstruction. Computed tomographic (CT) scans were available for 7 and 14 asthmatic subjects with and without chronic persistent obstruction, respectively. The average number of airways with an internal perimeter of greater than 0.6 cm sampled was 25.1 per subject. The parameters of interest were the lumen area, the wall area percentage, which is defined as the percentage of the airway area as seen in cross-section on a CT slice that is

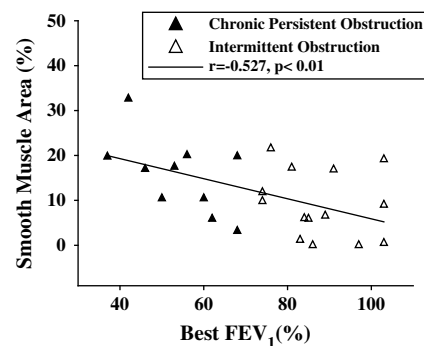


FIG 2. Correlation between SMA (percentage of total biopsy area) and FEV₁ (percent predicted) in subjects with severe asthma. *Solid triangles*, Subjects with chronic persistent obstruction; *open triangles*, subjects with intermittent obstruction.

occupied by the airway wall; and Pi10, which is the square root of the wall area for arbitrary hypothetical airway with an internal perimeter of 10 mm, as obtained from a regression equation based on data for each subject. No statistically significant differences between the groups were found (see Table E2 in this article's Online Repository at www.jacionline.org). There was also no significant correlation between the above HRCT-derived parameters and any of the histopathologic features of remodeling (Fig 3).

DISCUSSION

In this study we demonstrate that chronic persistent airflow obstruction in subjects with severe asthma is associated with an earlier age of onset and longer disease duration. There is ongoing inflammation with sputum eosinophilia and neutrophilia despite high doses of inhaled, and in some cases oral, corticosteroids. Most notably, we find evidence of greater degrees of airway remodeling in this patient group, particularly in the form of increased ASM and a trend toward increased subepithelial fibrosis. However, sputum cytokine and MMP analyses, biomarkers of inflammation and remodeling, respectively, do not distinguish this population from other subjects with severe asthma and neither do airway thickness parameters on HRCT.

Severe asthma is a heterogeneous disease¹⁷ and includes a subset of patients with fixed airflow limitation. In the current study we identified such patients based on the *a priori* defined cutoff point of an FEV₁ of 70% of predicted value, which is in accordance with ATS criteria for severity.⁸ We recognize that this arbitrary division might misclassify some subjects with mild degrees of chronic persistent airflow obstruction. Nonetheless, the selection process led to strikingly different degrees of airflow obstruction in the 2 groups and revealed significant differences in outcomes.

ASM mass is known to be increased in asthmatic subjects with longer disease duration,¹⁸ in subjects with fatal asthma,⁵ and in subjects with persistent severe asthma⁶ and has the potential to have important functional consequences. The basis for more extreme forms of airway remodeling is unknown but in some cases might reflect the additive effects of a slowly progressive and time-dependent process. In others it might be a manifestation of a distinct asthma subtype. Specific genetic variants have recently been associated with childhood-onset asthma and poor asthma control.^{19,20} The present findings raise the question of whether these variants could also be associated with specific patterns of airway

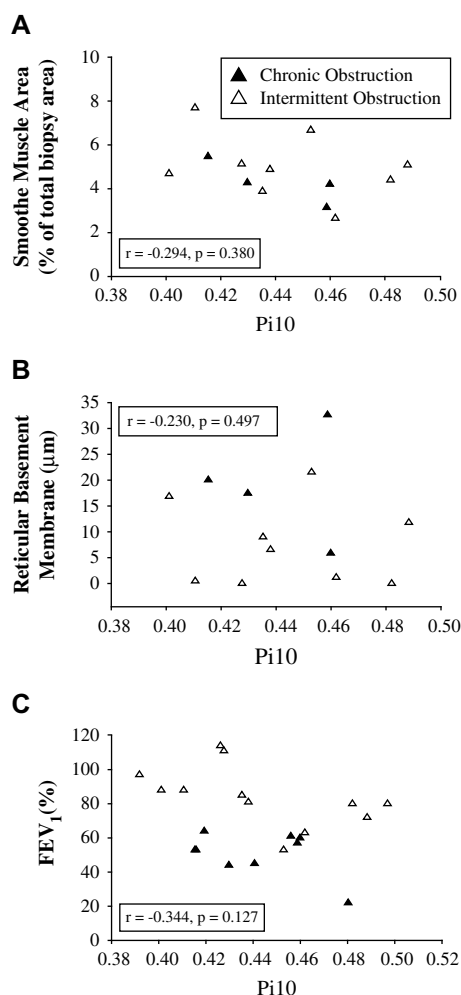


FIG 3. Correlation between Pi10 (a measure of airway wall thickness on HRCT, see the [Methods](#) section for details) and RBM thickness (**A**) and SMA % (**B**) for 13 subjects who underwent HRCT and a biopsy and between Pi10 and FEV₁ percent predicted (from the study visit closest to HRCT; $n = 21$; **C**).

remodeling. Indeed, in children with severe asthma, those with persistent obstruction had increased ASM compared with those without, despite similar age of onset and asthma duration,²¹ suggesting that the remodeling can occur in a relatively short time and remain in those in whom fixed obstruction is an associated feature.

The association between fixed airway obstruction and increased ASM was not expected. Even though modeling experiments predict that greater muscle thickness allows the development of greater airway wall tension, overcoming intrinsic impedances to airway narrowing and causing more airway constriction,²² adequate bronchodilator and anti-inflammatory therapy might be expected to reverse the obstruction. There was evidence of persistent inflammation, as reflected in sputum differential cell counts and increased FeNO levels, supporting the idea of persistence of stimuli for ASM shortening. However, both our subject groups had substantial inflammation. Another potential mechanism for the pattern of obstruction might lie in the ASMs capacity to adapt to various lengths by rearranging the length-tension relationship so as to optimize force generation, a phenomenon known as plasticity²³ or adaptation.²⁴ The muscle might

then become more difficult to stretch²⁵ and perhaps relatively fixed at short lengths.

Alternatively, ASM remodeling might be a marker for remodeling of other tissues and perhaps in more distal airways than those sampled. We had anticipated differences in the degree of airway wall fibrosis that might be expected to cause contracture of the airways or to prevent airway dilation induced by tidal breathing or deep breaths.²⁶ Submucosal fibrosis did show a trend toward an increase in the chronically obstructed group, although not a significantly one. The assessment of fibrosis by using histologic scoring techniques is intrinsically less quantitative than the assessment of ASM area by means of morphometry, and this might account for the uncertainty of the outcome, as could the smaller sample size. Furthermore, if fibrosis is of a greater degree in the peripheral airways, it could account for fixed airflow obstruction. Although epithelial damage is another frequently reported marker of remodeling, we observed no difference in the degree of epithelial detachment between groups and no association with extent of remodeling. The RBM thickness was also similar in the 2 groups. It is possible that the substantial steroid use in our study subjects, especially in those with chronic persistent obstruction, might have reduced epithelial damage and RBM thickening.^{27,28} However, our results are consistent with those of Tillie-Leblond²¹ in a pediatric population.

The noninvasive assessment of airway remodeling remains problematic. We attempted to measure airway thickness on HRCT as a marker of remodeling. However, no differences between groups were found, and there was no correlation between wall thickness and FEV₁, RBM thickness, or SMA ([Fig 3](#)). Although the power to detect a difference was low in this study, our findings are consistent with the findings of Little et al.²⁹ A correlation between RBM thickness and airway wall thickness has been reported in a group of adult subjects with mild-to-moderate asthma³⁰ but not in children.³¹ Of note, treatment with inhaled steroids decreases wall thickness,^{32,33} an effect that might be reflected in our findings. The apparent contradiction in the literature regarding HRCT in subjects with asthma^{29,34-36} might be due to different patient populations sampled, disease duration, treatment history, and different measurement techniques.

A limitation of our HRCT data is that 2 different scanners were used. Scanning all subjects by using the same protocol or by using a phantom was not feasible. However, the most important feature of the HRCT scan is the reconstruction algorithm,³⁷ and for this study, comparable high-resolution algorithms were used (Siemens = B60f, GE = Bone; see the [Methods](#) section in this article's Online Repository). Only medium-sized airways were analyzed because there is too much error in airways with an internal perimeter of less than 6 mm.³⁷ Although some investigators have focused on only 1 airway, Kasahara et al³⁰ demonstrated that thickening in subjects with asthma occurred to the same extent across different cross-sectional levels of the lung. Therefore we think that the measurements that we report here are appropriate and true reflections of the airway anatomy in our subjects, and in consequence, HRCT cannot be used as a noninvasive surrogate for ASM remodeling. Remodeling of ASM might alter airway mechanical properties without changes in wall thickness by reducing airway distensibility, which has been linked to airway remodeling,³⁸ but was not found to correlate with airway measurements on HRCT.³⁹ Only 1 study examined the relationship between distensibility and remodeling,⁴⁰ looking at RBM thickness in subjects using only salbutamol, a population quite different from ours.

We have found that the subjects with chronic persistent obstruction had higher levels of neutrophils and eosinophils combined. The ongoing inflammation, rather than “burned-out” disease, suggests that if remodeling is a detrimental result of inflammation, it might be a dynamic process and therefore reversible with appropriate control of inflammation. This remains to be proved for the changes in ASM but has already been shown for the RBM and the vascular component.^{27,33}

We measured several mediators in sputum supernatants to explore, in a preliminary manner, molecular determinants of active remodeling. ECP and IL-5 levels correlated with SMA, suggesting that eosinophilic (T_H2) inflammation might favor SMA remodeling, as it might favor other aspects of remodeling.⁴¹ IL-13, another T_H2 cytokine known for its proremodeling effects on ASM,⁴² also correlated with SMA. Interestingly, we also found an association between SMA and the T_H1 cytokines IL-12 and IFN- γ . The overall pattern of cytokine expression in the group with chronic persistent obstruction was confirmed by means of principal component analysis. However, this part of the study was exploratory and should be regarded as hypothesis generating. Although the role of the T_H1 response and of IFN- γ in particular in remodeling is not clear, IFN- γ expression is increased in subjects with severe asthma,¹² it inhibits ASM proliferation,⁴³ and it seems to have other effects that are opposite to IL-13⁴⁴ and possibly protective against excessive airway constriction. The relative balance of T_H1 and T_H2 cytokines might be the determinant of the ASM responsiveness and its propensity for remodeling.

We examined MMP-9 and TIMP-1 because they are believed to be involved in remodeling and both are expressed by ASM,^{45,46} but we found no difference between the groups. It is possible that the addition of the mucolytic dithiothreitol during sputum processing decreased recovery of MMP-9 and TIMP-1.⁷

In summary, we show that subjects with severe asthma with chronic persistent obstruction have earlier disease onset and longer disease duration. They have more remodeling of ASM and possibly submucosal fibrosis. ASM remodeling does not correlate with airway thickness on HRCT but is associated with ongoing inflammation. Although subjects with severe asthma with chronic persistent airflow obstruction appear clinically distinct from those without, it remains unclear whether these are 2 pathophysiologically and genetically distinct asthma subtypes or whether they belong to a continuum of disease severity and progression. Recent reports regarding bronchial thermoplasty, a treatment directed at reducing ASM mass in subjects with asthma,⁴⁷ are very interesting in view of our findings. Asthmatic subjects with chronic persistent obstruction with increased ASM might benefit most from the procedure.

We thank Mrs Lucero Castellanos for the quantification of inflammatory markers in sputum samples.

Clinical implications: New treatment strategies are needed for severe asthma with persistent airflow limitation and should be aimed at both inflammation and airway wall remodeling.

REFERENCES

1. Antonicelli L, Bucca C, Neri M, De Benedetto F, Sabbatani P, Bonifazi F, et al. Asthma severity and medical resource utilization. *Eur Respir J* 2004;23:723-9.

2. Noonan M, Chervinsky P, Busse W, Weisberg S, Pinna J, de Boisblanc B, et al. Fluticasone propionate reduces oral prednisone use while it improves asthma control and quality of life. *Am J Respir Crit Care Med* 1995;152:1467-73.
3. Nakagawa T, Hoshino M. Airway remodeling in asthma: an introduction. *Clin Rev Allergy Immunol* 2004;27:1-2.
4. Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P, et al. Differences in airway remodeling between subjects with severe and moderate asthma. *J Allergy Clin Immunol* 2005;116:544-9.
5. Carroll N, Elliot J, Morton A, James A. The structure of large and small airways in nonfatal and fatal asthma. *Am Rev Respir Dis* 1993;147:405-10.
6. Benayoun L, Druilhe A, Dombret M-C, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167:1360-8.
7. Kelly M, Keatings V, Leigh R, Peterson C, Shute J, Venge P, et al. Analysis of fluid-phase mediators. *Eur Respir J* 2002;20(suppl):24S-39S.
8. Proceedings of the ATS Workshop on Refractory Asthma. Current understanding, recommendations, and unanswered questions. *Am J Respir Crit Care Med* 2000;162:2341-51.
9. Lemiere C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, et al. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. *J Allergy Clin Immunol* 2006;118:1033-9.
10. Pizzichini E, Pizzichini M, Efthimiadis A, Evans S, Morris M, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308-17.
11. 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* 2005;171:912-30.
12. Shannon J, Ernst P, Yamauchi Y, Olivenstein R, Lemiere C, Foley S, et al. Differences in airway cytokine profile in severe asthma compared to moderate asthma. *Chest* 2008;133:420-6.
13. Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998;12:811-5.
14. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, et al. Eosinophil-associated TGF- β 1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1997;17:326-33.
15. Maghni K, Lemiere C, Ghezzi H, Yuquan W, Malo JL. Airway inflammation after cessation of exposure to agents causing occupational asthma. *Am J Respir Crit Care Med* 2004;169:367-72.
16. R Development Core Team (2008). R: a language and environment for statistical computing. R Foundation for Statistical Computing; 2008.
17. Miller MK, Johnson C, Miller D, Deniz Y, Bleecker E, Wenzel S, et al. Severity assessment in asthma: an evolving concept. *J Allergy Clin Immunol* 2005;116:990-5.
18. Bai TR, Cooper J, Koelmeyer T, Pare PD, Weir TD. The effect of age and duration of disease on airway structure in fatal asthma. *Am J Respir Crit Care Med* 2000;162:663-9.
19. Moffatt MF, Kabesch M, Liang L, Dixon A, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* 2007;448:470-3.
20. Tavendale R, Macgregor D, Mukhopadhyay S, Palmer C. A polymorphism controlling ORMDL3 expression is associated with asthma that is poorly controlled by current medications. *J Allergy Clin Immunol* 2008;121:860-3.
21. Tillie-Leblond I. Airway remodeling is correlated with obstruction in children with severe asthma. *Allergy* 2008;63:533-41.
22. Lambert RK, Codd SL, Alley MR, Pack RJ. Physical determinants of bronchial mucosal folding. *J Appl Physiol* 1994;77:1206-16.
23. Pratushevich VR, Seow CY, Ford LE. Plasticity in canine airway smooth muscle. *J Gen Physiol* 1995;105:73-94.
24. Bai TR. On the terminology for describing the length-force relationship and its changes in airway smooth muscle. *J Appl Physiol* 2004;97:2029-34.
25. Wang L, Pare PD, Seow CY. Plasticity in skeletal, cardiac, and smooth muscle: selected contribution: effect of chronic passive length change on airway smooth muscle length-tension relationship. *J Appl Physiol* 2001;90:734-40.
26. Pellegrino R, Sterk P, Sont J, Brusaco V. Assessing the effect of deep inhalation on airway calibre: a novel approach to lung function in bronchial asthma and COPD. *Eur Respir J* 1998;12:1219-27.
27. Chetta A, Zanini A, Foresi A, Del Donno M, Castagnaro A, D'Ippolito R, et al. Vascular component of airway remodeling in asthma is reduced by high dose of fluticasone. *Am J Respir Crit Care Med* 2003;167:751-7.
28. 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. *Am J Respir Crit Care Med* 1999;159:1043-51.
29. Little SA, Sproule MW, Cowan MD, Macleod KJ, Robertson M, Love JG, et al. High resolution computed tomographic assessment of airway wall thickness in chronic asthma: reproducibility and relationship with lung function and severity. *Thorax* 2002;57:247-53.
 30. Kasahara K, Shiba K, Ozawa T, Okuda K, Adachi M. Correlation between the bronchial subepithelial layer and whole airway wall thickness in patients with asthma. *Thorax* 2002;57:242-6.
 31. Saglani S, Papaioannou G, Khoo L, Ujita M, Jeffery P, Owens C, et al. Can HRCT be used as a marker of airway remodelling in children with difficult asthma? *Respir Res* 2006;7:46.
 32. Niimi A, Matsumoto H, Amitani R, Nakano Y, Sakai H, Takemura M, et al. Effect of short-term treatment with inhaled corticosteroid on airway wall thickening in asthma. *Am J Med* 2004;116:725-31.
 33. Capraz F, Kunter E, Cernik H, Ilvan A, Pocan S. The effect of inhaled budesonide and formoterol on bronchial remodeling and HRCT features in young asthmatics. *Lung* 2007;185:89-96.
 34. Gono H, Fujimoto K, Kawakami S, Kubo K. Evaluation of airway wall thickness and air trapping by HRCT in asymptomatic asthma. *Eur J Respir Dis* 2003;22: 965-71.
 35. Niimi A, Matsumoto H, Amitani R, Nakano Y, Mishima M, Minakuchi M, et al. Airway wall thickness in asthma assessed by computed tomography. Relation to clinical indices. *Am J Respir Crit Care Med* 2000;162: 1518-23.
 36. Boulet L, Belanger M, Carrier G. Airway responsiveness and bronchial-wall thickness in asthma with or without fixed airflow obstruction. *Am J Respir Crit Care Med* 1995;152:865-71.
 37. Nakano Y, Whittall K, Kalloger S, Coxson H, Pare P. Development and validation of human airway analysis algorithm using multidetector row CT. *Proceedings of SPIE* 2002;4683:460-9.
 38. Brown NJ, Salome CM, Berend N, Thorpe CW, King GG. Airway distensibility in adults with asthma and healthy adults, measured by forced oscillation technique. *Am J Respir Crit Care Med* 2007;176:129-37.
 39. Brown RH, SN, Mudge B, Diemer FB, Permutt S, Togias A. High-resolution computed tomographic evaluation of airway distensibility and the effects of lung inflation on airway caliber in healthy subjects and individuals with asthma. *Am J Respir Crit Care Med* 2001;163:994-1001.
 40. Ward C, Johns DP, Bish R, Pais M, Reid DW, Ingram C, et al. Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma. *Am J Respir Crit Care Med* 2001;164:1718-21.
 41. Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, et al. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 2003;112:1029-36.
 42. Bosse Y, Thompson C, Audette K, Stankova J, Rola-Pleszczynski M. Interleukin-4 and interleukin-13 enhance human bronchial smooth muscle cell proliferation. *Int Arch Allergy Immunol* 2008;146:138-48.
 43. Amrani Y, Tliba O, Choubey D, Huang CD, Krymskaya VP, Eszterhas A, et al. IFN-gamma inhibits human airway smooth muscle cell proliferation by modulating the E2F-1/Rb pathway. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L1063-71.
 44. Hakonarson H, Maskeri N, Carter C, Grunstein M. Regulation of TH1- and TH2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. *J Clin Invest* 1999;103:1077-87.
 45. Elshaw SR, Henderson N, Knox A, Watson S, Buttle D, Johnson S. Matrix metalloproteinase expression and activity in human airway smooth muscle cells. *Br J Pharmacol* 2004;142:1318-24.
 46. Liang K-C, Lee C-W, Lin W-N, Lin C-C, Wu C-B, Luo S-F, et al. Interleukin-1beta induces MMP-9 expression via p42/p44 MAPK, p38 MAPK, JNK, and nuclear factor-kappaB signaling pathways in human tracheal smooth muscle cells. *J Cell Physiol* 2007;211:759-70.
 47. Cox G, Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC, et al. Asthma control during the year after bronchial thermoplasty. *N Engl J Med* 2007;356:1327-37.

METHODS

Asthma severity

For inclusion in the study, patients had to fulfill at least 1 major and at least 2 minor criteria as follows.^{E1} Major criteria were (1) treatment with daily oral steroids for more than 50% of the previous 12 months and (2) treatment with high-dose inhaled steroid (>1,000 mg of fluticasone or equivalent per day) and at least 1 other add-on therapy (long-acting β -agonist, leukotriene receptor antagonist, or theophylline) continuously over the previous 12 months. Minor criteria were (1) need for daily short-acting β -agonist, (2) persistent airflow obstruction (prebronchodilator FEV₁ <70% of predicted value and FEV₁/forced vital capacity ratio <80% of predicted value), (3) 1 or more emergency care visits in the last 12 months, (4) 3 or more steroid bursts in the last 12 months, (5) prompt deterioration with 25% or lower dose reduction of oral corticosteroids, and (6) near-fatal asthma event in the last 3 years.

Compliance

All subjects took an inhaler of fluticasone and salmeterol (250/50 mg; Advair; GlaxoSmithKline, London, United Kingdom). During a 1-month run-in period, an electronic device, the MDILog (Medtrac Technologies, Lakewood, Colo), was attached to the inhaler and registered its use. We included only subjects who took the prescribed 4 inhalations per day on at least 70% of days during the run-in period and were thus considered to be sufficiently compliant to be considered to have refractory asthma in the face of adequate treatment.

Exclusion criteria

Subjects were excluded if they had any other known pulmonary disease, including chronic obstructive pulmonary disease, or any major comorbid disease that might affect asthma disease activity, such as HIV, metastatic cancer, and congestive heart failure.

Procedures

Symptom severity was graded according to the Juniper Asthma Control Questionnaire.^{E2} Spirometry was performed according to ATS standards.^{E3} Allergy skin prick tests with commercial extracts from common allergens were performed with the modified prick method; results were regarded as positive if the wheal was greater than 3 mm. Sputum was induced by using inhalations of increasing concentrations (3%, 4%, and 5%) of hypertonic saline and processed as previously described.^{E4} Exhaled NO levels were measured from sample bags by using the chemiluminescence technique with a Nitric

Oxide Analyzer (Sievers 280i; Sievers Instruments, Inc, Boulder, Colo) interfaced through an analog-to-digital converter board to a personal computer. Histologic image analysis was performed with the Image Pro-Plus 4.0 system (Media Cybernetics, Silver Spring, Md). Images were recorded on a CCD color video camera (Sony) mounted on a conventional light microscope (Olympus Optical Co). The program was used to analyze the percentage of intact epithelium and SMA and RBM thickness.

HRCT

All CT scans were acquired in the volume scan mode at suspended full inspiration without the use of intravenous contrast media while the subject was in the supine position with a Siemens Somatom +4 (Siemens AG Medical Solutions, Erlangen, Germany) or GE CTI (General Electric Medical Systems, Milwaukee, Wis). The airway analysis was performed with the 1-mm-thick or 1.25-mm-thick CT images and the high spatial frequency reconstruction algorithm (GE = Bone, Siemens = B60f). All airways cut in a reasonable cross-section (long/short internal diameter ≤ 2.2) were analyzed. Every fifth CT slice was used to avoid the possibility of airway overlap. Airway dimensions were measured with EmphyxlJ. A region of interest is selected by clicking in the general area where the airway is located; this causes the program to magnify that region. A seed point is placed in the airway lumen, and 64 rays are projected 360° around the airway. The x-ray attenuation values are measured along each ray, and the airway wall area is defined by using the full-width-at-half-maximum principle. The airway lumen and outer airway wall perimeter are measured by connecting the end points of each of the rays through the airway wall, and manual editing of projection rays is used to remove rays that project beyond the airway wall into neighboring dense structures, such as pulmonary arteries. The lumen area (Ai) is defined as the area inside the internal perimeter. The total area of the airway (Ao) is defined as the area inside the outer perimeter. The airway wall area is defined as the area between these 2 perimeters, and the wall area percentage (WA%) is determined as follows: $Ao - Ai / Ao \times 100$. Because WA% is dependant on airway size, smaller airways have a larger WA% than larger airways. A size-independent measure of airway wall thickness was created by plotting the internal perimeter of an airway against the square root of the wall area for each subject. Then the wall area of an arbitrary airway with an airway lumen of 10 mm (Pi10) is obtained for each individual by using the regression line.^{E5} Only airways with an internal perimeter of at least 6 mm were used for this analysis because previous data suggest that the error in the airway wall measurements in smaller airways is too large to be considered reliable.^{E6}

REFERENCES

- E1. Proceedings of the ATS Workshop on Refractory Asthma. Current understanding, recommendations, and unanswered questions. *Am J Respir Crit Care Med* 2000; 162:2341-51.
- E2. Juniper EF, O'byrne PM, Ferrie PJ, King DR, Roberts JN. Measuring asthma control. Clinic questionnaire or daily diary? *Am J Respir Crit Care Med* 2000; 162:1330-4.
- E3. American Thoracic Society. Standardization of spirometry, 1994 update. *Am J Respir Crit Care Med* 1995;152:1107-36.
- E4. Pizzichini E, Pizzichini M, Efthimiadis A, Evans S, Morris M, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154: 308-17.
- E5. Bosken CH. Small airway dimensions in smokers with obstruction to airflow. *Am Rev Respir Dis* 1990;142:563-70.
- E6. Nakano Y, Whittall KP, Kalloger SE, Coxson HO, Pare PD. Development and validation of human airway analysis algorithm using multidetector row CT. *Proceedings of SPIE* 2002;4683:460-9.

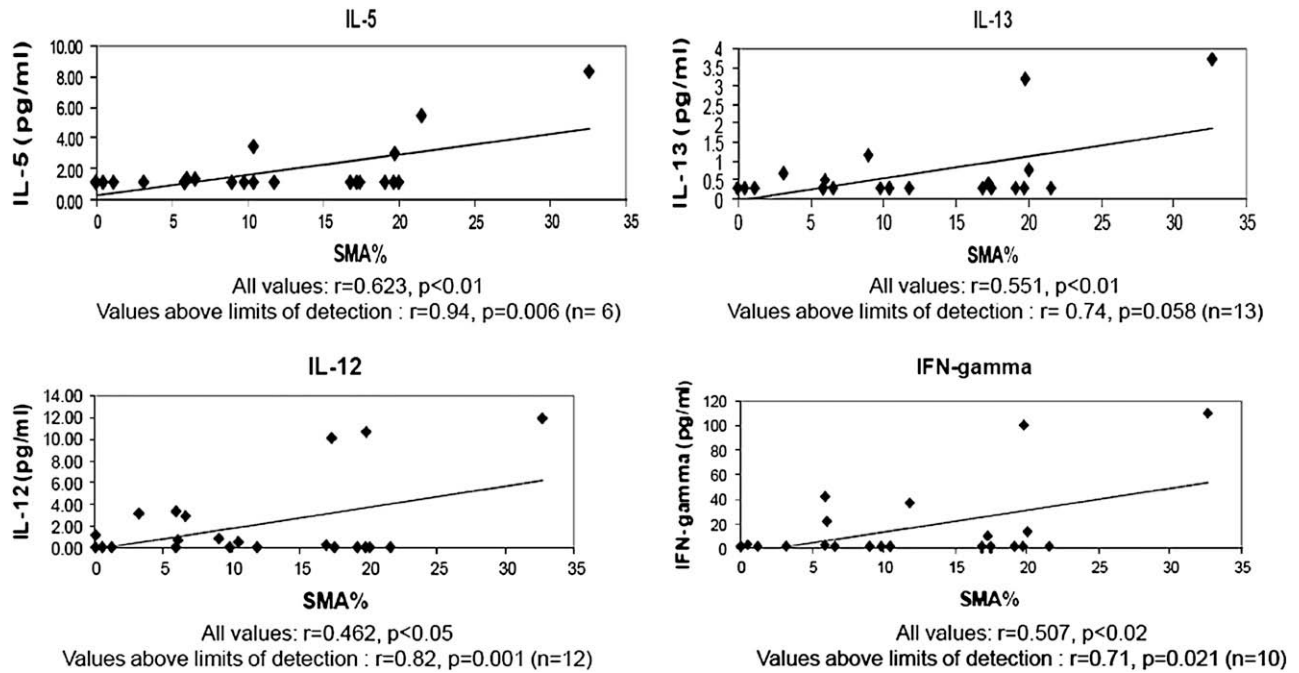


FIG E1. Correlation between SMA% on bronchial biopsy and concentrated sputum supernatant cytokines measured with the Bio-Plex assay.

TABLE E1. Values for cytokines and chemokines in concentrated sputum samples as measured with the Bio-Plex system: mean, minimal, and maximal values for the 2 groups of subjects with severe asthma

	Chronic persistent obstruction (n = 9)			Intermittent obstruction (n = 14)		
	Mean	Minimal	Maximal	Mean	Minimal	Maximal
Hu IL-1 β	18.38	1.84*	86.74	39.235	1.84*	143.23
Hu IL-1RA	4,760.23	268.51	24,752.76	6,093.941	33.92	25,158.23
Hu IL-2	0.88	0.10*	3.70	0.10*	0.10*	0.10*
Hu IL-4	0.88	0.33*	2.17	0.55	0.33*	2.41
Hu IL-5	2.39	1.11*	8.36	1.45	1.11*	5.47
Hu IL-6	3.68	0.10*	16.00	4.38	1.60	26.35
Hu IL-7	9.6	1.37*	35.81	4.62	1.37*	24.13
Hu IL-8	917.72	2.00*	4,253.49	2,587.32	2.00*	26,823.23
Hu IL-9	2.74	1.99*	5.19	4.05	1.99*	17.79
Hu IL-10	1.02	1.00*	1.12	1.26	1.00*	3.04
Hu IL-12 (p70)	3.04	0.10*	11.92	1.45	0.10*	10.12
Hu IL-13	1.07	0.28*	3.68	0.37	0.28*	1.15
Hu IL-15	0.99	0.56*	1.99	1.09	0.56*	4.77
Hu IL-17	2.34	1.82*	5.97	7.46	1.82*	39.64
Hu eotaxin	9.24	0.20*	21.6	6.35	0.20*	44.23
Hu FGF basic	2.20*	2.20*	2.20*	3.10	2.20*	14.73
Hu G-CSF	16.04	0.61*	52.02	36.68	0.61*	304.40
Hu GM-CSF	3.66	1.48*	7.05	5.94	1.48*	30.16
Hu IFN- γ	26.14	1.84*	109.36	9.37	1.84*	42.07
Hu IP-10	1,177.61	4.58*	4,838.21	1,390.92	4.58*	9,014.59
Hu MCP-1	7.71	0.01*	30.61	74.03	0.01*	938.63
Hu MIP- α	3.42	1.20*	14.78	5.69	1.20*	31.01
Hu MIP- β	81.16	0.40*	436.80	162.76	0.40*	1,198.85
Hu PDGFbb	17.08	2.90*	56.88	23.59	2.90*	199.64
Hu RANTES	1.07	0.76*	3.52	19.97	0.76*	259.75
TNF- α	2.00	1.72*	3.23	2.04	1.72*	4.16
VEGF	116.90	9.20	254.40	260.55	3.36	1,664.28

All values are presented in picograms per milliliter.

Hu, Human; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; IP-10, IFN- γ -induced protein 10; PDGFbb, platelet-derived growth factor bb; VEGF, vascular endothelial growth factor.

*Levels less than the detection limit of the assay.

TABLE E2. HRCT airway data (all airways with lumen perimeter >0.60 cm)

	Chronic persistent obstruction (n = 7)	Intermittent obstruction (n = 14)	<i>P</i> value
Wall area (%)	72.61 ± 0.94	71.08 ± 0.96	1
Pi10	0.4486 ± 0.00818	0.4391 ± 0.00881	.97
Lumen area (cm ²)	0.11 ± 0.026	0.11 ± 0.041	.91
Total area (cm ²)	0.3676 ± 0.0473	0.3635 ± 0.0762	.95

No statistically significant differences between groups were shown (Wilcoxon rank sum test).

Pi10, Wall area of an arbitrary airway with an airway lumen of 10 mm as calculated from a linear regression.