Specificity of basement membrane thickening in severe asthma

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Background: Reticular basement membrane (RBM) thickness is considered a hallmark for airway remodeling in airway diseases such as asthma. It is still unclear whether this measurement could be associated with disease severity or apply to chronic obstructive pulmonary disease (COPD). A wide range of results, at baseline or after therapeutic intervention, have been reported using different measurement methods.
Objective: To determine whether increased RBM thickness could be associated specifically with severe asthma and in COPD in large samples.
Methods: We blindly measured RBM thickness in endobronchial biopsies from 50 patients with severe asthma (mean age, 53 years; FEV_1 66% predicted, inhaled steroids ≥1500 μg and 20 mg daily dose of oral corticosteroids, lifelong nonsmokers), 50 untreated patients with mild asthma (mean age, 33 years; FEV_1 93%pred, lifelong nonsmokers), 50 patients with COPD (mean age, 57 years; FEV_1 53%pred, all current smokers), and 18 control subjects using 2 different validated quantitative and computer-assisted methods (repeated multiple point-to-point vs area by length ratio).
Results: Reticular basement membrane thickness was higher in severe asthma compared with mild asthma and COPD ($P = .0053$). On the basis of receiver operating characteristic curves, RBM thickness was effective in differentiating severe asthma from other groups (sensitivity and specificity, 98% and 95%, respectively, above a threshold of 5 μm vs control, 70% and 75% at 7 μm vs mild, 83% and 68% at 6 μm vs COPD).
Conclusion: Increased RBM thickness was specifically associated with severe asthma, whereas surprisingly, COPD and mild asthma had similar remodeling features.
Clinical implications: Reticular basement membrane thickness can be considered a hallmark of severe asthma. (J Allergy Clin Immunol 2007;119:1367-74.)

Key words: *Asthma, COPD, remodeling, basement membrane, ROC*

Abbreviations used
AUC: Area under curve
COPD: Chronic obstructive pulmonary disease
EBB: Endobronchial biopsy
RBM: Reticular basement membrane
ROC: Receiver operating characteristic
TEM: Transmission electron microscopy

Asthma is a chronic inflammatory disorder of the airways that leads to recurrent episodes of airway obstruction. Some structural changes can occur in association with inflammation, such as mucous gland hypertrophy, enlargement of bronchial smooth muscle, and collagen matrix deposition. This remodeling underlies chronic airflow obstruction. Thickening of the reticular basement membrane (RBM) has been attributed to excessive collagen deposition beneath the true basement membrane, especially involving subepithelial myofibroblasts and T-cell activation.[1,2] James et al[3] demonstrated in autopsy samples that RBM thickness correlated with the whole bronchial thickness, suggesting that a change in RBM thickness is a hallmark of the remodeling process. Some relationships with functional parameters that are thought to be linked with severity, such as FEV_1 or, more often, PC_{20} methacholine, have sometimes been reported.[4] The specificity of this phenomenon is still unknown, especially because it has also been reported in chronic obstructive pulmonary disease (COPD).[5]

Reticular basement membrane thickness is widely used in clinical and therapeutic studies as a marker of airway remodeling. A broad range of RBM thickness values, from 3 to 25 μm,[6-9] has been reported in controls and patients with asthma. Various techniques have been used, from transmission electron microscopy (TEM) to specific immunoassays of RBM components.[8] Sullivan et al[9] reported that multiple point-to-point repeated measurements at regular 20-μm intervals were correct when 40 measurements per slide were performed. On the other hand, Wilson and Li[8] described satisfactory results in hematoxylin-eosin–stained biopsies with RBM assessment on the basis of the area by length ratio, compared with using TEM measurements as a gold standard. In most of these studies, the number of cases investigated was quite low and the clinical relevance questionable.

Reticular basement membrane thickness may be difficult to assess because of fixation and embedding artefacts, as well as technical bias caused by the reorientation requirements, and irregularities.[8,10] Stereologic approaches reduce such bias,[10] but they are time-consuming and limit the potential of studies involving large patient populations.

We hypothesized that increased RBM thickness could be specific enough to discriminate between severe asthma and other chronic airway diseases, including milder forms

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Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.
Received for publication May 17, 2006; revised January 19, 2007; accepted for publication January 24, 2007.
Available online May 4, 2007.
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0091-6749/$32.00
© 2007 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2007.01.055

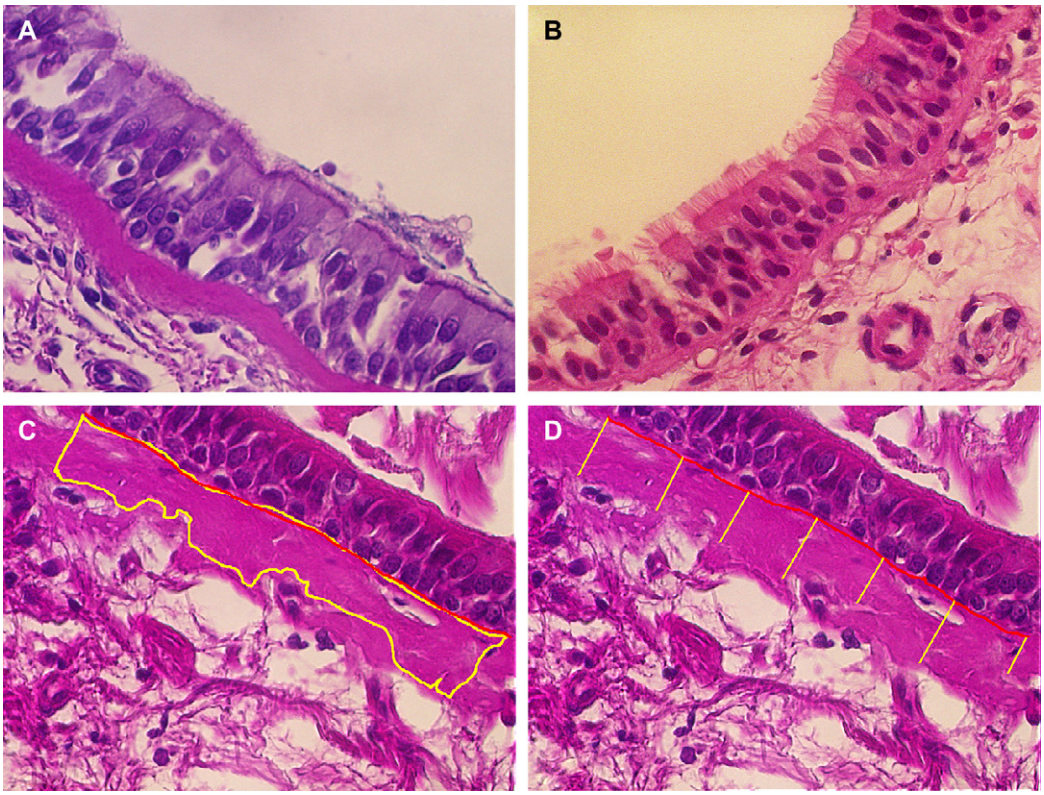


FIG 1. Different examples obtained in this study. A patient with mild asthma **(A)** and COPD **(B)**. RBM thickness measurements in a patient with severe asthma according to Wilson **(C)** and Sullivan **(D)**.

of the disease. To test this hypothesis, we used 2 different validated methods involving light microscopy to obtain precise data with large patient populations.

METHODS

Patients

From 1996 to 2000, endobronchial biopsies (EBBs) were performed in 6 different clinical studies, approved by ethics committees, involving more than 300 patients. We selected the first 50 well characterized patients with mild-to-moderate untreated asthma, 50 current smokers with untreated COPD, and 50 patients with severe oral steroid–treated asthma. The diagnoses were made according to current guidelines.[11,12] The first 2 groups of patients were naive for anti-inflammatory treatment at the time of sampling. Flow-volume curves were assessed at baseline and after inhaling 400 μg salbutamol via a spacer. Atopic status was assessed by skin prick tests for allergens from the Montpellier area. Smoking status was checked through patient interviews. All patients with asthma had a clear history of recurrent wheezing or breathlessness episodes that reversed spontaneously or after treatment, a familial history of asthma, and at least 15% reversibility after bronchodilator inhalation[11] or a positive methacholine provocation test with a PC_{20} <4 mg/mL. Active smokers or exsmokers of more than 5 pack-years were excluded. Asthma severity was based on medication requirements (high daily dose of inhaled corticosteroids >1500 μg/d, long-acting β_2 agonists, and in some cases, oral corticosteroids at required doses) and permanent airflow impairment in patients followed up for at least 1 year, as previously defined.[13,14] Disease onset was recorded on the basis of patient interviews or medical files when available. Patients with COPD had a clear history of smoking, chronic productive cough or sputum, no history of asthma even in their family, no reported allergic diseases, and no significant reversibility after bronchodilator treatment. All patients with COPD had a high-resolution computed tomography scan in their files confirming the presence of centrolobular emphysema at the parenchymal level. All patients were stable for at least 1 month before the investigation. Eighteen slides of tissues from normal volunteer subjects stored in our tissue databank were used as controls to compare the results.

EBB sampling and selection

Between 3 and 6 EBBs were performed on 1 occasion, with flexible bronchoscopy under local anesthesia, in the left lower lobe with crocodile forceps according to recommendations.[15] After fixation in neutral 4% formaldehyde buffer and embedding in paraffin, each biopsy was sliced and then stained with hematoxylin-eosin. Four to 6 sections were fixed on a slide for subsequent analysis.

Randomization

Each slide was twice randomly assigned a number according to 2 independent centralized lists. First, a single observer assessed the RBM thickness of all endobronchial biopsies by multiple point-to-point repeated measurements at regular intervals of 20 μm according to Sullivan et al.[9] Then the observer assessed the RBM thickness by the area by length ratio according to Wilson and Li[8] (Fig 1). Measurements were performed over 1 mm while taking only the adequately preserved and oriented area into account. Metaplastic as well as fully shed areas were considered inadequate for measurement.

The intraobserver and intramethod reproducibilities were assessed on 40 randomly chosen slides. All measurements were made with a computerized image analyser (AnalySIS 3.0 for Windows,

TABLE I. Patient characteristics*

	Mild (n = 50)	Severe (n = 50)	COPD (n = 50)	Controls (n = 18)	*P* value
Male (%)	64	57	88	50	NS
Age (y)	33 (26-49.75)	53 (39-62)	60 (51.5-66.75)	26 (22-34)	.003
Postbronchodilator FEV_1 (%pred)	94.5 (79.25-100)	66 (51.50-74)	59 (45.25-67.75)	100 (97.25-104)	.02
Reversibility (%)	13 (11.5-18)	17 (14-22)	4 (0-9)	2 (0-3)	.0001
Smoking (pack-years)	0 (0-0.75)	0 (0-1)	40 (40-50)	0 (0-1.25)	.00004
Atopy (%)	70	62	0	0	.003
Disease duration (y)	8 (2-12)	8.4 (3-15)	9 (2-18)	NA	NS
Inhaled corticosteroids (μg/d)	0	2000 (1500-3000)	0	NA	.002

NA, Not applicable; *NS*, not significant.
*Comparisons were made using the Kruskal-Wallis test with adequate posttest corrections. Values are means with 95% CIs for Gaussian variables, and median with 25 and 75 interquartiles for others.

Olympus Soft Imaging Solutions, Muenster, Germany) linked to a CCD camera (Sony CCD DXC950P, Sony, Tokyo, Japan) connected to a light microscope at ×400 magnification (Olympus BHS, Olympus Optical, Tokyo, Japan). The pixel resolution was approximately 0.02 μm.

Assessment

Reticular basement membrane thickness was expressed as the geometric mean of repeated measurements by using the Sullivan method or as the average area to length ratio by using the Wilson method, as previously recommended.[8,9]

Statistical analysis

Categorical variables were summarized as percentages and continuous variables as means and SDs for Gaussian variables and medians with interquartile ranges for non-Gaussian variables.

The *t* test and ANOVA or the Wilcoxon and Kruskal-Wallis tests were used for comparing continuous variables between groups, according to whether their distribution was Gaussian.

Correlations between functional or clinical continuous variables were estimated with the Spearman rank ρ index.

Intramethod agreement was evaluated with the Bland and Altman[16] method and the intraclass correlation coefficient.

Receiver operating characteristic (ROC) curves were used to evaluate the validity of basement membrane thickening measurements for discriminating between airway diseases. Their areas under curve (AUCs) with 95% CIs were computed.[17]

In the current study, equal weight was given to sensitivity and specificity. Cut-points that optimize the differentiating ability of the basement membrane thickening were estimated with the Youden[18] index, which is the maximum vertical distance between the ROC curve and the diagonal or chance line. Sensitivity and specificity were computed according to these optimal cut-points. *A posteriori* power calculations were performed with an α risk at 5% and a power of 80% and 90%.

The statistical significance was set at $P < .05$. As sequential tests were performed, we corrected the *P* value with the Bonferroni correction to maintain the overall 2-sided type 1 error rate at 5%.

The computations were performed with the SAS software package (version 8.2; SAS Institute Inc, Cary, NC).

RESULTS

Patient characteristics

Patients with mild and severe asthma and COPD presented markedly different phenotypes, as reported in Table I. Seventy percent of patients with mild asthma and 60% with severe asthma were atopic, and 3 of them were exsmokers (<5 pack-years, ceased for at least 1 year; Table I). Patients with COPD had a lower reversibility than patients with asthma ($P = .0001$). They were all current heavy smokers. Severe asthma and COPD groups presented with similar lung function at rest but significantly different reversibility after bronchodilator inhalation. No differences between groups were noted in terms of disease duration, whereas only patients with severe asthma patients were receiving high doses of inhaled corticosteroids.

TABLE II. RBM thickness in asthma and COPD, according to both techniques

	Wilson		Sullivan	
	Mean (μm)	SD	Mean (μm)	SD
Controls (n = 18)	4.16	0.66	4.95	0.47
Mild asthma (n = 50)	5.63*	2.03	6.85*	1.99
COPD (n = 50)	4.80*	2.12	5.68*	1.90
Severe asthma (n = 50)	6.25*†	2.10	7.23*†	1.97

*$P < .0001$ vs controls.
†$P < .0001$ vs mild asthma and COPD (Kruskal-Wallis with the adequate Bonferroni and Dunn posttest corrections).

Assessment of RBM thickness

Reticular basement membrane thickness presented a normal distribution in all groups and homosedasticity among the severe asthma, mild asthma, and COPD groups. Mean RBM thickness was statistically lower ($P < .001$) in the control group than in the 3 others (Table II). Slides not adequately reversed or oriented for accurate measurements were excluded (3, 4, 6, and 2 slides per group, respectively).

With both methods, RBM was statistically thicker in severe asthma (mean, 7.23 μm according to Sullivan; 6.25 μm according to Wilson) than in mild asthma (mean, 6.85 μm according to Sullivan, $P = .0002$; 5.63 μm according to Wilson, $P < .0001$), in COPD biopsies (mean, 5.68 μm according to Sullivan, $P < .0004$; 4.80 μm

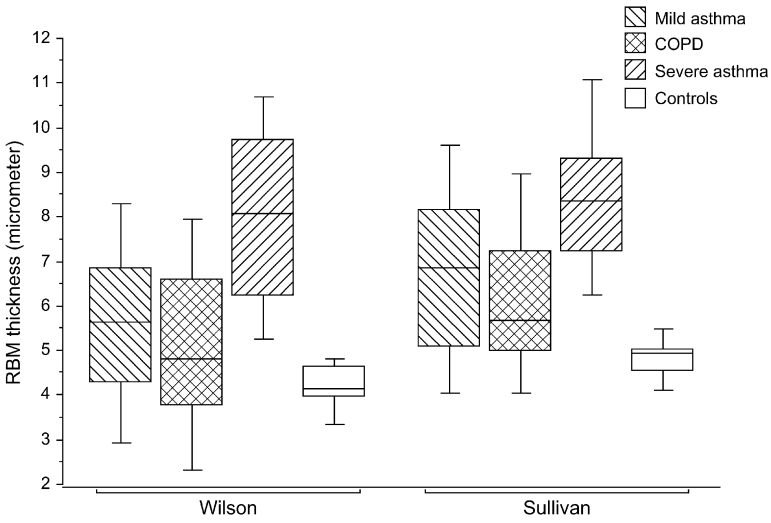


FIG 2. Box-plot distribution according to the 2 techniques.

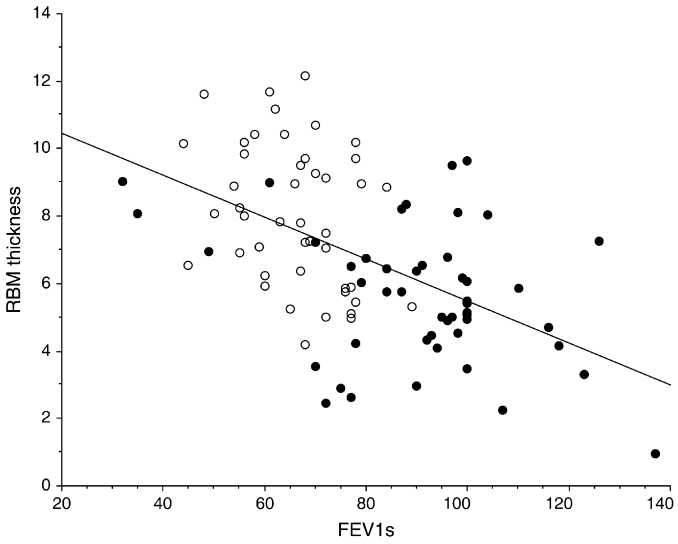


FIG 3. Spearman correlation between RBM thickness (μm) and postbronchodilator FEV_1 (% of predicted value) in mild *(solid circles)* and severe *(open circles)* asthma. $\rho = -0.56$; $P < .0001$.

according to Wilson, $P < .0001$), or in control biopsies (mean, 4.95 μm according to Sullivan, $P = .0004$; 4.16 μm according to Wilson, $P < .0001$; Table II).

No significant differences were noted between mild asthma and COPD, irrespective of the method used (Fig 2). *A posteriori* power calculations revealed that 150 to 300 subjects per group would be necessary to detect a statistically significant difference between mild asthma and COPD.

Reticular basement membrane was also thicker in the mild asthma group than in the control group (mean, 4.83 μm with Sullivan, $P = .0004$; 4.23 μm with Wilson, $P = .016$). Thickening was also encountered in the COPD group compared with the control with the Sullivan method ($P = .0004$). A trend toward RBM thickening was also noted when the Wilson method was used ($P = .06$).

The results were always higher with the Sullivan method compared with the Wilson method (paired *t* test, $P < .0001$ for the 4 groups).

We did not find any correlation with any clinical or functional parameters in mild and severe asthma, or in COPD (Spearman rank ρ index for FEV_1 and RBM thickness in the severe asthma group, $\rho = 0.224$; $P = .343$). A significant correlation between FEV_1 and RBM thickness was observed ($\rho = -0.56$, $P < .0001$ according to Wilson; $\rho = -0.51$, $P < .0001$ according to Sullivan; Fig 3) when considering mild and severe asthma together (Fig 3). Duration of the disease and RBM thickness correlate neither within nor among groups.

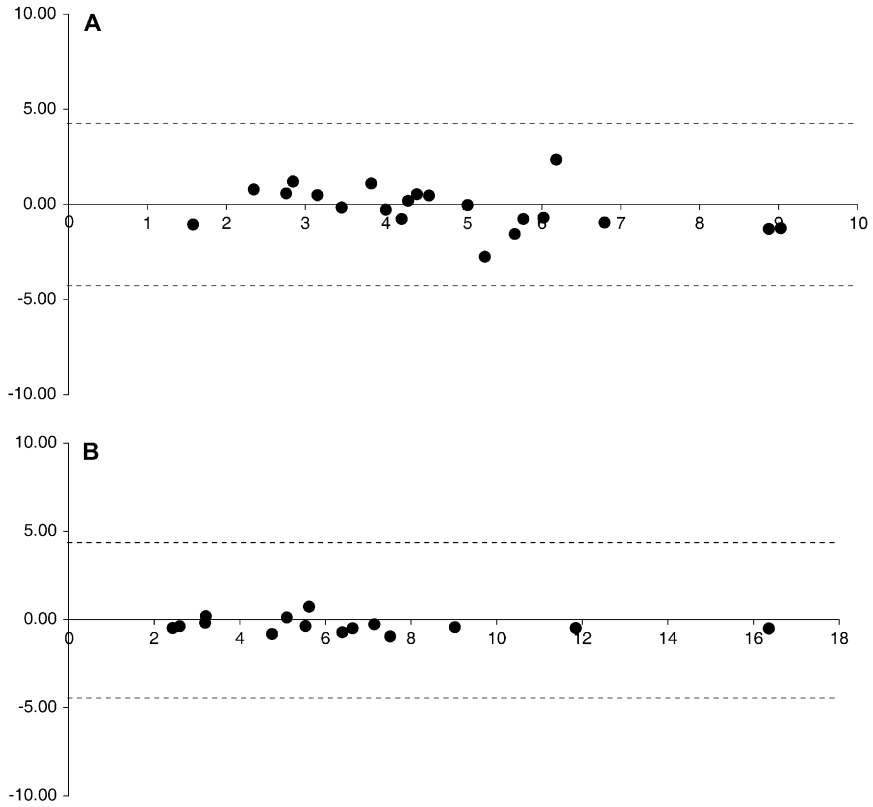


FIG 4. Bland and Altman graphs for reproducibility in Wilson's **(A)** and Sullivan's **(B)** techniques (intraobserver).

TABLE III. Values of the area under a ROC with 95% CI and *P* values[14]

	Wilson		Sullivan	
	AUC	***P* value**	**AUC**	***P* value**
Mild vs control	0.751 (0.630-0.872)	.002	0.798 (0.685-0.911)	.0003
COPD vs control	0.655 (0.511-0.801)	.058 NS	0.756 (0.629-0.884)	.002
COPD vs mild	0.574 (0.455-0.694)	.226 NS	0.596 (0.470-0.723)	.143 NS
Severe vs control	0.978 (0.945-1)	10^{-9}	0.964 (0.922-1)	$<10^{-9}$
Severe vs mild	0.790 (0.690-0.882)	10^{-6}	0.735 (0.632-0.840)	.0002
Severe vs COPD	0.838 (0.754-0.922)	10^{-8}	0.815 (0.724-0.906)	10^{-7}

Reproducibility

Both methods were highly reproducible according to the Bland and Altman[16] visual analysis (Fig 4). The intraclass correlation coefficient was 0.84 (95% CI, 0.66-0.94) for RBM measurement according to Wilson and 0.81 according to Sullivan (95% CI, 0.67-0.89).

ROC curves

We quantified the ability of RBM thickness to discriminate between diseases with AUC computed from ROC curves. AUC differentiated severe asthma from mild asthma, COPD, and control biopsies (Table III). The RBM thickness differentiated severe asthma from any group among the 3 others: the AUC was 0.790 (95% CI, 0.698-0.883) for severe versus mild asthma, 0.838 (95% CI, 0.754-0.922) for severe asthma versus COPD, and 0.963 (95% CI, 0.92-1) for severe asthma versus controls (Fig 5).

The detection cutoff for severe asthma versus mild asthma (or COPD), which maximized the Youden index, was 7 μm and 6 μm, respectively. When these cutoffs were used, the sensitivity and specificity were 70% and 75%, respectively, when comparing severe and mild asthma, and 83% and 68% when comparing severe asthma and COPD (Table IV).

Because the cost of false-negatives and false-positives may be considered as similar in these diseases, we determined the cutoffs for pathology detection by maximizing the Youden index. The results are presented in Table IV. The cutoff values shifted with the technique. When comparing severe asthma to mild asthma, COPD, and controls, these cutoffs were associated with high sensitivity and specificity of RBM thickening—that is, 98% and 95%, respectively—at a threshold of about 5 μm versus controls, 70% and 75% at 7 μm versus mild asthma,

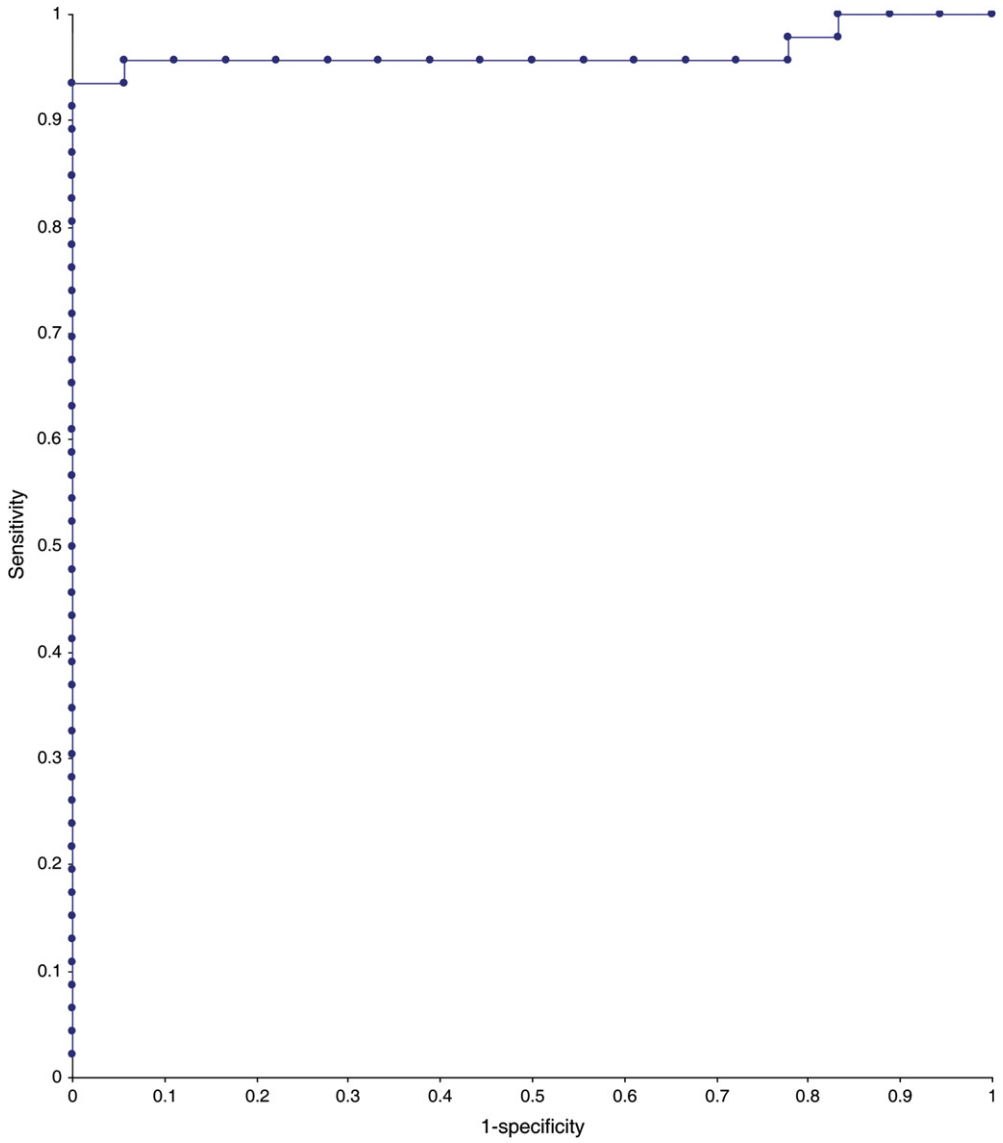


FIG 5. ROC graph illustrating the ability of RBM measurement to discriminate between controls and severe asthma according to the Wilson technique (sensitivity, 98%; specificity, 94.4% at a threshold of 4.96 μm).

and 83% and 68% at 6 μm versus COPD, according to Wilson's method (Table IV).

DISCUSSION

This study demonstrated that RBM thickness could differentiate patients with chronic airway disease when measured by 2 different validated methods. Remodeling in severe asthma was clearly different from remodeling in mild asthma and COPD, both of which differed from the situation observed in control subjects.

We chose the methods for RBM assessment by Wilson and Li[8] and Sullivan et al[9] because of their wide use and validation compared with methods as effective as TEM.[8] Stereologic approaches have demonstrated their superiority in this respect, but they are time-consuming and difficult to use with large patient populations.[10] Moreover, our biopsies were not processed for it. We decided to use 2 different methods to check our approach. The results gave satisfactory results with similar differences among groups. Comparing methods was not the aim of this study. Clearly, these 2 methods are not identical, nor do they measure the same structure, because the absolute results were different. The area-to-length ratio method enables more observer bias to delineate the possible nonlinear upper or lower RBM limits, whereas the repeated measurements of Sullivan's method provide a more accurate indication on possible variations in RBM thickness. The presence of capillary or inflammatory cells infiltrated within the basement membrane can be subtracted with Wilson's method, whereas this cannot be done with Sullivan's method because of the regular intervals required. The reproducibility of the methods assessed by

TABLE IV. Sensitivity and specificity of increased RBM thickness for discriminating between groups, provided by the ROCs

	Wilson			Sullivan		
Condition	**Threshold (μm)**	**Sensitivity (%)**	**Specificity (%)**	**Threshold (μm)**	**Sensitivity (%)**	**Specificity (%)**
Mild vs control	4.92	66.6	94.4	5.88	71	100
COPD vs control*	5.05	49	94.4	5.38	67.5	88.8
Mild vs COPD*	4.8	51	66.6	6.72	70.3	53.7
Severe vs control	4.96	98	94.4	5.94	93.5	100
Mild vs severe	6.91	69.5	75	7.04	82.6	61
COPD vs severe	5.86	82.6	68	6.65	89	67.6

*Results not statistically significant and reported for informational purpose only.

the Bland and Altman[16] graphical presentation and the intraclass coefficient of correlation assessment was as high as in other studies.

We ensured that the patients included in our study were clearly representative of well-described phenotypes to avoid overlapping forms of these diseases. Borderline conditions were thus avoided as much as possible. The patient populations studied here were much larger than those investigated in all other studies on the same topic. Nonetheless, the patients with the most severe asthma could not be safely tested with a methacholine provocative test, as could be done with patients with severe COPD. Otherwise, the tobacco smoking status was based on patient interviews.

Other confounding risk factors for increased RBM thickness such as diabetes mellitus[19] were controlled before inclusion. The absence of discrimination between mild asthma and COPD, instead of being different from controls, is likely to induce many comments, because the patients' phenotypes were at the ends of the clinical spectrum. Age differences could not account sufficiently for RBM thickness differences.[19] First, it could be argued that the remodeling process may also occur at the proximal level in COPD, even though the results differed between techniques, with different mechanisms involved. This has been recently suggested by others.[20] Otherwise, increased RBM thickness in posttransplantation bronchiolitis or interstitial pulmonary fibrosis has already been reported.[21] The excessive ECM deposition that occurs under the true RBM could be interpreted as nonspecific visual evidence of healing processes in response to different deleterious phenomena. Very few studies have been conducted on COPD endobronchial biopsies focused on RBM thickness. New studies focused on this aspect are required to investigate remodeling more precisely on proximal airways in COPD. The nature and amounts of extracellular matrix products involved could differ from those observed in asthma.[20] Increased collagen content and small airway fibrosis have been reported in COPD, while the molecular nature of extracellular matrix deposition and cellular sources were not clearly identified.[12,20]

Severe asthma is more likely to induce such a pathological process, thus supporting the idea that remodeling increases with the disease severity. No correlations could be clearly observed within our groups, but phenotypic heterogeneity is a feature of severe asthma.[13] Patients with permanent airflow obstruction represent only 1 phenotype of severe asthma. For instance, a positive relationship between RBM thickness and PC_{20} methacholine was controversially reported in the past.[4,22,23] Moreover, the patients with severe asthma involved in this study were all receiving heavy treatments, including inhaled and oral steroids, that might affect RBM thickness to largely unknown extents. Recent clinical studies that used RBM thickness as a marker of therapeutic intervention against excessive airway remodeling reported that inhaled steroids tended to decrease RBM thickness.[6,24,25] On the other hand, some *in vitro* studies suggested that steroids could increase extracellular matrix deposition.[26] Nonetheless, whereas the patients with the most severe asthma had the highest RBM thicknesses, in the current study we noted, like Minshall et al[27] in a previous study, a negative correlation between RBM thickness and FEV_1 when considering the 2 asthma groups. In a variable and heterogeneous condition such as severe asthma, a single parameter such as FEV_1 did not account for the complexity of the disease. Jeffery[20] demonstrated that the true basement membrane was not affected by the remodeling process. The organization of the fibers in the thickened RBM in asthma, contrary to what is expected in fibrosis, does not resemble that of interstitial collagen,[28] suggesting a qualitative more than a quantitative pathway. The more severe disease then leads to more severe remodeling because duration of the disease judged by patient's interview was not different.

Irrespective of these potential biases, because our ROC curves sustained our results, such RBM thickening could thus be considered as a real feature associated with severe asthma. The exact potential role of this remodeling process—that is, protective against excessive airway narrowing[29] or responsible for chronic airflow impairment[20,30]—should be investigated in more in-depth studies. Patients with severe asthma and COPD presented the same airflow impairment but different RBM thicknesses, as demonstrated before,[31] suggesting a disease specific process rather than a simple association with a nonspecific airway remodeling mechanism.

Safety issues can be considered as potential limits but observing recommended cautions permit researchers to obtain optimal results.[32] In this study, we did not encounter any safety issues, because all patients requiring

bronchoscopy had been previously investigated and eventually excluded according to international guidelines.[15] This is a potential limiting factor with respect to investigating the patients with the most severe disease.

By comparison with the current study, the exhaled NO level has been studied as a diagnostic tool for asthma, and the results obtained in these studies were not as good as those obtained with methacholine provocation—that is, around 75% sensitivity and specificity.[33] Although the CI was higher than in controls, a RBM thickness assessed visually in a quasi-routine setting could be used as a diagnostic criterion for severe asthma, with positive predictive values that are higher than exhaled NO.[33]

In conclusion, this study is the first to demonstrate the potential discriminative diagnostic benefits of routine RBM thickness assessment, at least in patients with severe asthma. These findings suggest that increased RBM thickness is related to the disease and its severity rather than nonreversible chronic airflow obstruction. Important questions still to be addressed concern differences in the remodeling process in severe asthma and COPD, and the functional and biological consequences of increased RBM thickening.

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