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ORIGINAL ARTICLE

The clinical utility of reticular basement membrane thickness measurements in asthmatic children

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Abstract

Objective: Reticular basement membrane (RBM) thickness is one of the pathological features of asthma and can be measured in endobronchial biopsies. We assessed the feasibility of endobronchial biopsies in a routine clinical setting and investigated the clinical value of RBM thickness measurements for asthma diagnosis in children. **Methods:** We included all children who underwent bronchoscopy with endobronchial mucosal biopsies for clinical reasons and divided them into three subgroups: (1) no asthma, (2) mild–moderate asthma, and (3) problematic severe asthma. **Results:** In 152/214 (71%) patients, mean age 9.5 years (SD 4.6; range 0.1–18.7) adequate biopsies were retrieved in which RBM thickness could be measured. Mean (SD) RBM thickness differed significantly among children without asthma, with mild–moderate asthma, and with problematic severe asthma ($p = 0.04$), 4.68 (1.24) μm , 4.56 (0.89) μm , and 5.21 (1.10) μm respectively. This difference disappeared after adding exhaled nitric oxide to the multivariate model. **Conclusions:** This study confirms the difference in RBM thickness between children with and without asthma and between asthma severities in a routine clinical care setting. However, quantifying the RBM thickness appeared to have no added clinical diagnostic value for asthma in children.

Keywords

Childhood asthma, endobronchial biopsy, exhaled nitric oxide, pediatrics, remodeling

History

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Introduction

In children with asthma, flexible bronchoscopy may provide useful information on co-morbidity or differential diagnosis [1]. Additionally, analysis of bronchoalveolar lavage (BAL) fluid gives information on microbiology, micro-aspiration and inflammatory cells in the airways, which may guide treatment decisions. In experienced hands, flexible bronchoscopy with BAL in children with asthma carries a negligible risk [2]. Endobronchial biopsies provide supplementary information on airway inflammation and features of remodeling [3,4], including reticular basement membrane (RBM) thickening. In this retrospective study in two centers (Erasmus Medical Centre – Sophia Children's Hospital and University Medical Centre Groningen – Beatrix Children's Hospital), we first aimed to assess the feasibility of endobronchial biopsies in children in a routine patient care setting. Second, we compared RBM thickness among non-asthmatic children,

children with mild-moderate asthma, and children with problematic severe asthma (PSA). We hypothesized that objective evidence of airway remodeling, as reflected by RBM thickness in biopsies, will be more pronounced in patients with – than in those without – asthma, and most abnormal in PSA. Third, we investigated the clinical value of RBM thickness measurement for asthma diagnosis. Some of the results of this study have been previously reported in the form of abstracts [5,6].

Materials and methods**Study population**

All children who underwent bronchoscopy for clinical reasons between January 2007 and May 2011 at Sophia Children's Hospital (Rotterdam, The Netherlands) or between January 2004 and December 2011 at Beatrix Children's Hospital (Groningen, The Netherlands), in whom endobronchial mucosal biopsies were taken routinely were included in this study. Based on data from electronic medical records, patients were divided into three mutually exclusive subgroups: (1) no asthma, (2) mild–moderate asthma, defined as doctor's diagnosis of asthma and well-controlled or partly controlled

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asthma according to GINA guidelines [7], (3) PSA, defined as doctor's diagnosis of asthma with uncontrolled symptoms despite the use of $\geq 800\mu\text{g}$ budesonide or equivalent dose of inhaled corticosteroids (ICS) and long acting beta-agonists, while adherence to treatment and inhalation technique were optimized and exposure to triggers avoided as much as possible. Atopy was defined as RAST class 2 or higher and/or positive skin prick test for at least one aeroallergen. Two independent reviewers (E. M. and M. P.) who were blinded for RBM thickness classified patients. The study was approved by the hospital's Medical Ethics Committees.

Study design

This was a retrospective study. Patient files were used to collect data on demographics, diagnoses, level of asthma control, spirometry (forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1), and forced expiratory flow at 75% of vital capacity (FEF75)), fraction of exhaled nitric oxide (FeNO), atopy, and medication use. These data were collected from the outpatient clinic visit closest to the date of bronchoscopy with a maximum window of 3 months. Lung function parameters and FeNO were measured after at least 48 h cessation of inhaled corticosteroids. Results of bronchoscopy, BAL fluid cultures, and cell differentiation were collected from electronic patient files.

Bronchoscopy, BAL, and biopsy processing

Bronchoscopies were performed under general anesthesia with a flexible fiberoptic bronchoscope (3.6 mm BF 3C30, 3.8 mm BF 3C160, or 4.9 mm BF P160, Olympus, Center Valley, PA). In children with asthma, inhaled bronchodilators were administered 30 min before the procedure. For safety reasons, most patients with PSA were pre-treated with oral or intramuscular steroids. BAL was performed in the right middle lobe with 1 ml/kg warm NaCl 0.9% (max 20 ml per aliquot), up to four times. Under direct view, endobronchial mucosal biopsies were taken from the right middle or lower lobe. Biopsy specimens were fixed in formalin and embedded in paraffin wax. Biopsy sections were cut at 3- μm thickness, mounted on microscopy slides and stained with hematoxylin-eosin (HE). These were digitalized and analyzed for RBM thickness with NanoZoomer Digital Pathology viewer software, Hamamatsu, Hamamatsu City, Japan (Rotterdam) or Aperio Image Scope viewing software, Vista, CA (Groningen). Only undamaged biopsy sections containing sub epithelial RBM were selected. Tangential sections were avoided. RBM thickness was measured in μm at 10 different locations throughout the biopsy and the mean of these 10 measurements was used for analyses (Figure 1). This 10 points method is fast, relatively easy to perform and there is no need for expensive software. We validated the 10 points method with the reported area method, in which the mean thickness of the RBM is derived by dividing the area of the RBM by the length of the RBM in an uninterrupted evaluable region of 100 μm (Figure 2) [8]. Within-biopsy and between-biopsy, variability of RBM thickness using the 10 points method was determined in a subset of 24 patients with at least two evaluable biopsies. Both intra- and interobserver variabilities were assessed in the same subset. All RBM

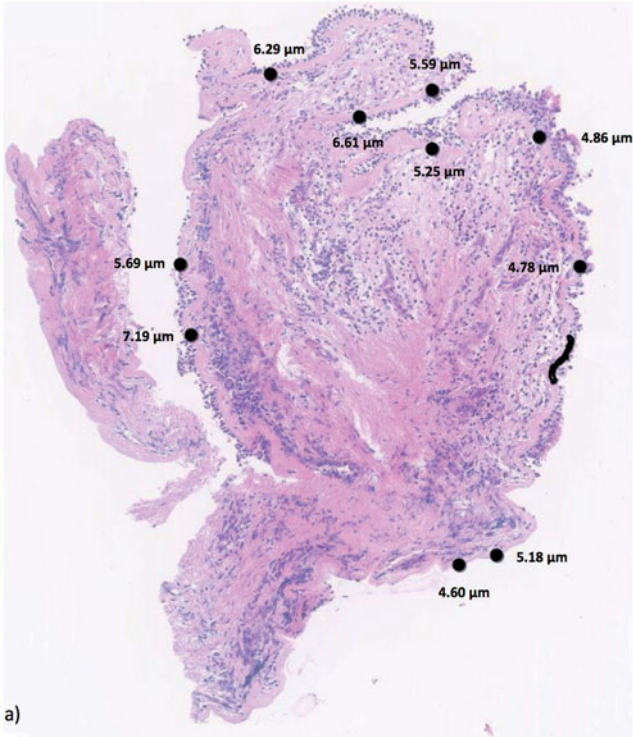


Figure 1. Reticular basement membrane thickness measurement in an endobronchial biopsy stained with hematoxylin-eosin (magnification 5 $\times$).

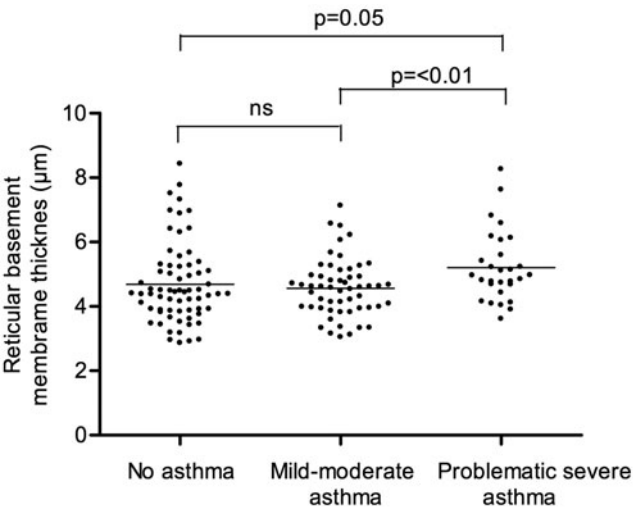


Figure 2. Reticular basement membrane thickness (μm) in children without asthma, children with mild-moderate asthma, and children with problematic severe asthma. ns, not significant.

thickness measurements were performed by two investigators (L. V. and F. H.) who were blinded for the clinical diagnosis.

Statistical analyses

Differences in baseline characteristics among non-asthmatic, mild-moderate asthmatic children, and children with PSA were assessed using ANOVA for continuous variables and the Chi-square test for categorical variables. FeNO values were log transformed to obtain a near normal distribution. We evaluated within-biopsy variability using the coefficient of

Table 1. Clinical characteristics.

	No asthma (n = 107)	Mild-moderate asthma (n = 70)	Problematic severe asthma (n = 37)	p Value
Mean age (years) ^a	8.9 (4.6)	9.7 (4.6)	11.0 (4.5)	0.06
Gender (male %)	44/107 (41%)	42/70 (60%)	18/37 (49%)	0.05
FVC (% predicted) ^a	98.5 (16.1) n = 80	97.9 (13.1) n = 54	97.1 (15.4) n = 34	0.89
FEV1 (% predicted) ^a	97.6 (17.6) n = 81	92.5 (16.0) n = 54	88.5 (21.1) n = 34	0.03
FEF 75 (% predicted) ^a	75.3 (29.9) n = 79	61.0 (28.8) n = 52	54.5 (34.1) n = 34	0.04
FeNO (ppb) ^b	8.8 (5.6–12.5) n = 64	13.1 (7.8–20.0) n = 52	23.1 (11.7–39.3) n = 32	< 0.01
Atopy (yes %)	20/107 (19%)	40/70 (57%)	30/37 (81%)	< 0.01
ICS use (yes %)	25/107 (23%)	56/70 (80%)	37/37 (100%)	< 0.01
BAL eosinophils (×10 ⁹ /L) ^a	3.6 (12.2)	4.3 (7.6)	3.7 (4.2)	0.93
BAL neutrophils (×10 ⁹ /L) ^a	20.0 (24.6)	15.2 (20.9)	17.8 (22.1)	0.56
BAL culture (positive %)	48/105 (46%)	30/70 (43%)	12/36 (33%)	0.43

FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; FEF75, forced expiratory flow at 75% of vital capacity; FeNO, fractional exhaled nitric oxide; ICS, inhaled corticosteroids; BAL, bronchoalveolar lavage. The ANOVA was used for continuous variables and the Chi-square test for categorical variables; *p* Values in bold indicates statistical significance <0.05.

^aData reported as mean (SD).

^bData reported as geometric mean (interquartile range).

Table 2. Indications for bronchoscopy.

Indications for bronchoscopy	Children without asthma (n = 107)	Children with asthma (n = 107)
Asthma with expected co-morbidities	n.a.	34
Therapy resistant asthma	n.a.	37
Suspected anatomical abnormalities	19	8
Recurrent infections	48	9
Bronchiectasis	9	2
Chronic cough	18	8
Other	13	9

variation (CV). Variability among methods, biopsies, and intra- and inter-observer variability were analyzed using intraclass correlation coefficients (ICC) and Bland and Altman plots [9]. Linear regression was used to analyze differences in RBM thickness between the three patient groups. We used multivariate models to investigate whether RBM thickness is an independent predictor for an asthma diagnosis. For all statistical analyses SPSS version 17.0 (SPSS Inc., Chicago, IL) was used.

Results

We included 214 patients (104 males), mean age 9.5 years (SD 4.6; range 0.1–18.7). One hundred and seven children were not asthmatic, 107 had asthma, of whom 37 were diagnosed as PSA. Clinical characteristics are shown in Table 1. The asthmatic children tended to be older and were more often male than in the non-asthma group. FEV1 and FEF75 were significantly lower; FeNO and atopy significantly higher in both asthma groups. Indications for bronchoscopy are presented in Table 2.

Feasibility biopsies

From the 214 patients, in our study in whom biopsies were attempted, we obtained on average 3.8 biopsies per patient that were evaluable in 152 children (71%). Reasons for non-evaluable biopsies were no material present at microscopy slides (*n* = 15), mechanically damaged material or no

recognizable RBM present (*n* = 33), RBM thickness was not evaluable (*n* = 14) due to either insufficient staining or tangential sections. We observed an increased success rate from 59% to 84% after we switched from re-usable forceps to disposable forceps in 2008. RBM thickness could be measured in 68 (64%) of the non-asthmatic children, 55 (79%) of the mild-moderate asthmatic children, and in 29 (78%) children with PSA. No serious or unexpected complications were reported during or after bronchoscopy.

Repeatability and variability of RBM thickness

Twenty-four patients had two or more evaluable biopsies and these were used for the variability analyses. Within single biopsies the coefficient of variation of 10 measurements of RBM thickness was on average 23% (range 11–46%). Within the same patient, the between biopsy variability of RBM thickness showed an ICC of 0.72 (95% CI: 0.53–0.85). Intra-observer variability for RBM thickness was excellent (ICC 0.95 [95% CI: 0.87–0.98]) and the inter-observer variability was good (ICC 0.90 [95% CI: 0.78–0.96]). Bland and Altman plots showed good agreement (Online supplement). In 66 biopsies, the mean RBM thickness measured with the 10 points method was compared with the mean RBM thickness measured with the area method, ICC was 0.88 (95% CI: 0.81–0.92) and Bland and Altman plot showed good agreement.

Comparison of RBM thickness between patient groups

Mean RBM thickness differed slightly but significantly between the three groups (*p* = 0.04, Figure 2). Mean RBM thickness was similar in non-asthmatic children and children with mild-moderate asthma (mean 4.68 μm and 4.56 μm, respectively, mean difference −0.12 μm, 95% CI: −0.51 to 0.27, *p* = 0.54). There was a significant difference in RBM thickness between children with mild-moderate asthma and children with PSA (mean 5.21 μm, mean difference 0.64 μm, 95% CI: 0.15 to 1.14, *p* = 0.01), and between children without asthma and those with PSA (mean difference 0.52 μm, 95% CI: 0.04 to 1.00, *p* = 0.03).

We found no correlation between RBM thickness and age, gender, atopy or cells in BAL fluid. FVC, FEV1, and geometric mean FeNO each correlated significantly with RBM thickness (Table 3). Age, gender, and atopy did not influence the association between RBM thickness and asthma diagnosis in the multivariate model, whereas adding FeNO completely abolished the difference in RBM thickness between children with PSA and children without asthma (mean difference 0.09 μm , 95% CI: -0.44 to 0.61 , $p=0.75$) (Table 4). Also, the difference between the mild-moderate asthma group and the PSA group disappeared after adding FeNO to the model (mean difference 0.36 μm , 95% CI: -0.13 to 0.84 , $p=0.15$). Adjustment for FEV1, but not for FVC, reduced the difference between the children without asthma and children with PSA, but the effect was much smaller than that of FeNO (Online supplement).

Discussion

This is the largest clinical study to date reporting on endobronchial biopsies in children in a routine clinical care setting. Children with PSA had a slightly thicker RBM than children without asthma and children with mild–moderate asthma. RBM thickness correlated with FeNO and the difference in RBM thickness between groups disappeared completely after adjusting for FeNO.

Increased RBM thickness has been found previously, both in preschool children with wheezing symptoms [10] and in children with asthma [11–15]. Recently, a summary of all bronchial biopsy studies in children was published [16]. Similar to the study of Chetta et al. [17], we found a

significant difference in RBM thickness between children with PSA and children with mild–moderate asthma. Other studies did not report a difference between asthma subgroups, which may be explained by different definitions of asthma severity [12,13]. The considerable overlap in RBM thickness may relate to the heterogeneity of asthma, to inhomogeneity of histological abnormalities within subjects and, hence, sampling error. A single assessment of RBM thickness is, therefore, probably not of diagnostic value in individual patients.

We found relatively normal RBM thickness in asthmatic children compared with previous reports [11,12,15]. One explanation could be that the administration of systemic steroids before bronchoscopy in children with PSA has reduced RBM thickness. This seems unlikely, however, as effects of steroids on RBM as documented in asthmatic adults are slow and take several months [18]. Indeed, there was no association between the use of ICS and oral or intramuscular steroids shortly before bronchoscopy and RBM thickness.

Sagiani et al. [19] found no evidence of RBM thickening in symptomatic, wheezing infants under the age of 2 years and, hence, any difference in RBM between asthmatic and non-asthmatic children in our study might have been obscured by including <2 year olds. However, this did not seem to be the case, because limiting our analyses to children >2 years of age, our results remained the same (data not shown).

We demonstrated a correlation between RBM thickness and FeNO, which has not been reported before [13,14,20]. Mahut et al. [21] did show a relationship between maximal NO output and RBM thickness in children with refractory asthma. In previous studies, the use of high dose inhaled or systemic corticosteroids before bronchoscopy may explain lack of correlation, as the effect of corticosteroids on FeNO may occur within days while effects on RBM take much longer [18]. FeNO is a biomarker of eosinophilic airway inflammation, and it could well be that the RBM thickness is increased as a result of long-standing effects of eosinophil-derived inflammatory mediators and cytokines on subepithelial deposition of collagen [22]. That RBM thickness relates to eosinophilic inflammation is further supported by our finding that adding FeNO to the multivariate model abolished the difference in RBM thickness between children with PSA and without asthma.

This study has several limitations. The diagnosis of asthma was based on retrospective evaluation of electronic patient

Table 3. RBM thickness: correlation with baseline characteristics.

	N	Spearman's correlation coefficient	p Value
Age (years)	152	0.009	0.91
Gender	152	−0.08	0.33
FVC (% predicted)	126	−0.23	0.01
FEV1 (% predicted)	127	−0.18	0.04
FEF75 (% predicted)	125	−0.11	0.24
log FeNO	113	0.45	< 0.01
BAL eosinophils ($\times 10^9/\text{L}$)	106	0.12	0.21
BAL neutrophils ($\times 10^9/\text{L}$)	108	0.13	0.19

FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; FEF75, forced expiratory flow at 75% of vital capacity; FeNO, fractional exhaled nitric oxide; BAL, bronchoalveolar lavage.

Table 4. Difference RBM thickness between groups.

	No asthma (reference)	Difference no asthma versus mild-moderate asthma	Difference no asthma versus problematic severe asthma	Subgroup
Model A (crude estimates)	4.68 (4.42–4.95)	−0.12 (−0.51 to 0.27) $p=0.54$	0.52 (0.04 to 1.00) $p=0.03$	RBM measurement available, $n=152$
Model B (crude estimates in subgroup)	4.63 (4.34–4.92)	−0.09 (−0.51 to 0.33) $p=0.68$	0.55 (0.07 to 1.03) $p=0.03$	RBM measurement and FeNO available, $n=113$
Model C (Model B with FeNO added)	4.81 (4.51–5.10) ^a	−0.27 (−0.68 to 0.14) $p=0.19$	0.09 (−0.44 to 0.61) $p=0.75$	

RBM, reticular basement membrane; FeNO, fractional exhaled nitric oxide. Data reference group presented as mean (95% confidence interval). Data mild–moderate and problematic severe asthma group presented as the mean difference from the reference group (95% confidence interval).

^aRBM thickness in reference group (no asthma) reported for average FeNO (13.2 ppb).

files. This precluded accurate standardized assessment of the pre- and post-test probability of an asthma diagnosis. To minimize the risk of misclassification, two independent and experienced physicians who were blinded for RBM thickness assessed all patients for an asthma diagnosis. The fact that FEV1 was significantly lower and FeNO higher in the asthma groups compared with non-asthmatic controls supports the validity of our classification. We could not include a true healthy control group due to ethical considerations; our control group did have respiratory symptoms which may have influenced RBM thickness and FeNO [23]. Furthermore, RBM thickness is only one aspect of airway remodeling and the importance of RBM thickness for the clinical phenotype of childhood asthma is unclear. Unfortunately, eosinophil counts on our HE stained sections revealed poor intra-observer and inter-observer repeatabilities and material for additional immunohistochemical staining was not available. Therefore, we also could not reliably quantify other aspects of remodeling such as epithelial loss, smooth muscle hypertrophy, and goblet cell hyperplasia in our retrospective analysis.

In conclusion, pediatric endobronchial biopsies were feasible and safe in a routine clinical care setting. In line with previous studies, we found a thicker RBM in children with PSA, but also a large overlap between children with and without asthma in this clinical setting. RBM thickness appeared to have limited diagnostic value over FeNO for asthma in children.

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Supplementary material available online
Supplementary material Online Supplement.