

Letter to the Editor

Expression of semaphorin 3E is suppressed in severe asthma*To the Editor:*

Asthma is a chronic inflammatory disease of the airways associated with bronchoconstriction, remodeling, and airway hyperresponsiveness (AHR). Deciphering mediators that are pathologically dysregulated in patients with asthma is a key step toward the development of more efficient therapeutic strategies, particularly for steroid-refractory severe patients.¹ Semaphorins are guidance cues that play diverse roles such as immune regulation and angiogenesis.² Emerging evidence suggests that semaphorin family members, for example, semaphorin 3A,³ semaphorin 4A,⁴ and semaphorin 7A⁵ contribute to the pathogenesis of allergic asthma. Semaphorin 3E (Sema3E) is a chemorepellent involved in essential cellular functions that are impaired in allergic asthma. Our previous studies demonstrated a reduction in the Sema3E receptor, PlexinD1, on human airway smooth muscle cells obtained from patients with asthma. Treatment of these cells with exogenous Sema3E inhibits their proliferation and migration, which are considered key features of airway remodeling in asthma.⁶ As such, we hypothesized that the airways of patients with allergic asthma display dysregulated Sema3E expression, which may contribute to airway remodeling.

We first determined the expression pattern of Sema3E in bronchial biopsies from patients with mild, moderate, and severe

allergic asthma as well as healthy donors. The clinical characteristics of the patients and healthy individuals are presented in Table E1 in this article's Online Repository at www.jacionline.org. Sema3E, as a secreted protein, was highly expressed in bronchial biopsies of healthy donors (Fig 1, A). Patients with mild (Fig 1, B) and moderate (Fig 1, C) allergic asthma showed a tendency toward reduced Sema3E expression although this was not statistically significant. As demonstrated in Fig 1, D, a robust reduction of Sema3E expression was observed in those with severe asthma. Staining with isotype control antibody confirmed the specificity of Sema3E immunoreactivity (Fig 1, E). These data suggest that Sema3E could be considered a novel mediator involved in the severity of allergic asthma.

We found that the major cells displaying Sema3E immunoreactivity in bronchial biopsies of healthy individuals include epithelial cells. Then, we examined whether Sema3E is down-regulated in asthmatic bronchial epithelial cells from patients with severe asthma. As demonstrated in Fig 2, A, basal Sema3E level, as detected by ELISA, was significantly lower in the primary epithelial cells obtained from patients with severe allergic asthma compared with those of healthy donors. Furthermore, analysis of quantitative PCR data demonstrated a significant downregulation of *Sema3e* gene expression in bronchial epithelial cells from patients with severe asthma (Fig 2, B) compared with cells from healthy individuals. Thus, we revealed that Sema3E expression is reduced in bronchial epithelial cells from patients with severe asthma.

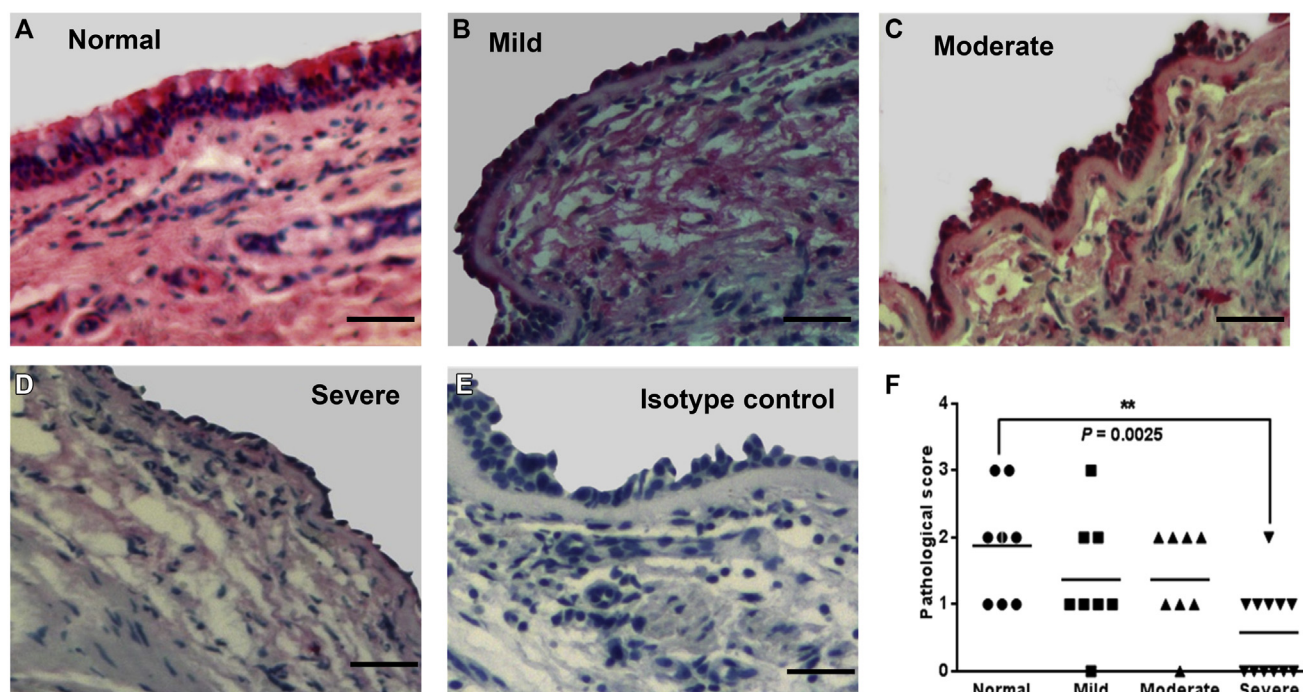


FIG 1. Expression of Sema3E is reduced in lung biopsies from patients with severe asthma. Expression of Sema3E was studied by performing immunohistochemistry on bronchial sections from healthy subjects (A) and from individuals with mild (B), moderate (C), and severe (D) asthma. E, Staining with isotype control showed no cross-reactivity. F, Sema3E expression was scored in a blind manner and statistically compared. Scale bars: 50 μ m.

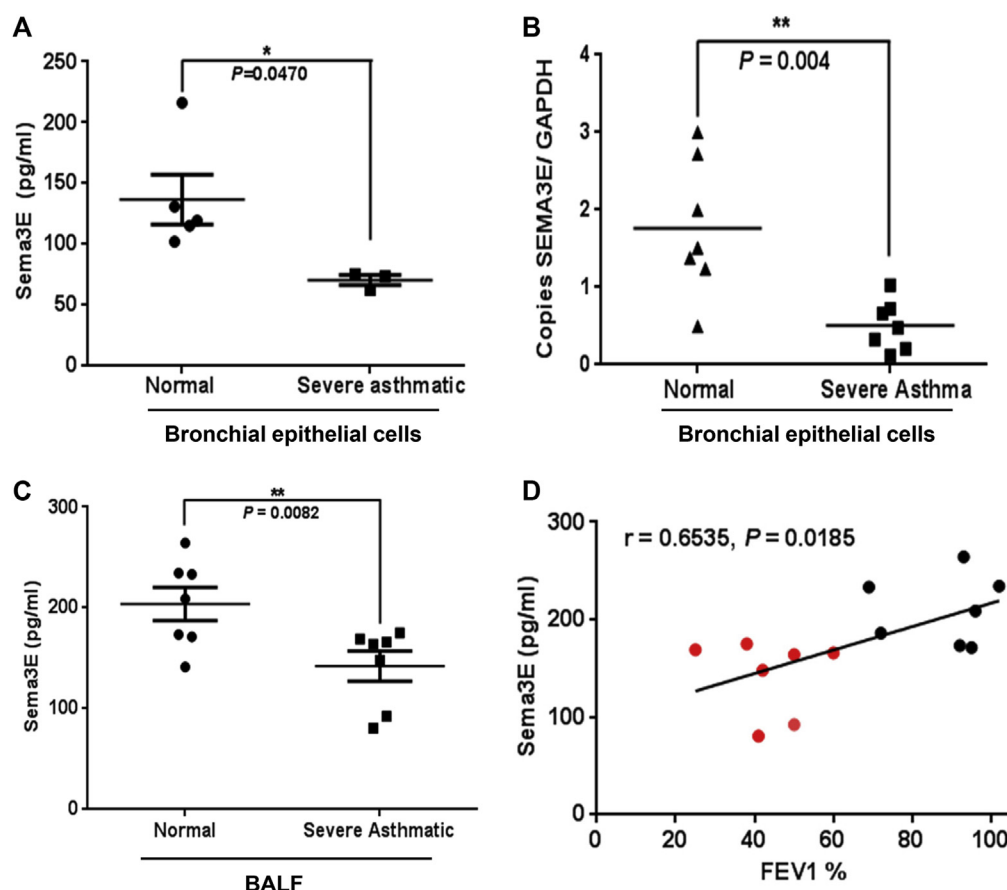


FIG 2. Sema3E is downregulated in epithelial cells and airways of patients with severe asthma. Supernatant and mRNA were obtained from bronchial epithelial cells to measure Sema3E expression by ELISA (**A**) and quantitative PCR (**B**), respectively. **C**, Sema3E secretion into the airways was studied by ELISA. **D**, Correlation of Sema3E level with FEV₁ was assessed by Pearson R test. Red and black dots represent subjects with severe asthma and healthy subjects, respectively.

Because the functional form of Sema3E is a secreted protein, we then studied *in vivo* secretion of Sema3E protein into the airways by comparing its level in human bronchoalveolar lavage fluid (BALF) obtained from healthy donors and patients with severe asthma by ELISA. The clinical characteristics of patients with severe asthma and healthy controls are presented in Table E2 in this article's Online Repository at www.jacionline.org. In line with our bronchial biopsies data, there was a significant decrease in Sema3E protein secretion into the airways of patients with severe asthma compared with healthy donors (Fig 2, C). Downregulation of Sema3E in bronchial epithelial cells from patients with severe asthma may account for its reduced level in the airways of patients with severe asthma as observed in BALF. We next investigated the potential correlation between Sema3E level and clinical measures of those with severe asthma. Our statistical analysis indicated that there is a strong inverse correlation between FEV₁ and Sema3E expression (Fig 2, D).

Collectively, our data demonstrate that Sema3E expression is decreased in severe allergic asthma, suggestive of a potential role of this molecule in regulating asthmatic phenotype.

Under asthmatic conditions, along with augmented epithelial inflammatory response,⁷ we found decreased expression of Sema3E in the airway epithelium. Therefore, it is plausible that Sema3E may negatively regulate the release of inflammatory

mediators from the epithelial cells and recruitment of inflammatory effector cells. To support this notion, we previously reported a repulsive effect of Sema3E on human (airway smooth muscle) cell functions *in vitro*.⁶ Also, our recently published data suggest that exogenous intranasal Sema3E treatment prevents allergic airway inflammation and AHR upon house dust mite allergen challenge *in vivo*.⁸ Furthermore, Sema3E-deficient mice exhibit exaggerated inflammatory response, AHR, and remodeling in acute house dust mite model of allergic inflammation.⁹ However, further investigations would prove the involvement of Sema3E in airway epithelial response during allergic asthma.

Bronchial biopsies specimens were obtained from the Institut Universitaire de Cardiologie et de Pneumologie de Quebec (IUCPQ) site of the Respiratory Health Network Tissue Bank of the FRQS (Quebec, Canada). Subjects with asthma fulfilling the American Thoracic Society criteria for asthma¹⁰ were recruited from the Asthma Clinic at the IUCPQ. Subjects' characteristics are summarized in Table E1. Subjects with mild asthma were atopic with at least 1 positive response to common allergens on allergy skin prick tests. They were nonsmokers and used only an inhaled β_2 -agonist on demand and did not use inhaled or systemic corticosteroids. In the month preceding the study, none of the subjects reported a respiratory infection or an increase in asthma symptoms. Subjects with severe asthma have

severe asthma according to the American Thoracic Society refractory asthma definition.¹⁰ Subjects were on a continuous treatment of high doses of inhaled corticosteroids (1000 µg of fluticasone propionate or the equivalent in combination with a long-acting β₂-agonist). They were nonsmokers, and their asthma was stable with no exacerbation for the last 4 months. Healthy subjects were nonatopic and nonsmokers with no history of asthma or atopy or any respiratory diseases. The study was approved by the IUCPQ (Quebec, Canada) ethics committee and all subjects signed an informed consent form.

Human BALF was obtained from subjects with severe asthma and healthy controls (Table E2) according to procedures approved by the Human Research Ethics Board of the Department of Medicine, University of Chicago (Institutional Review Board nos. 13198A and 15361A) following the receipt of the subjects' written informed consent. Pulmonary function tests were performed with methacholine challenge before their surgery. All bronchoscopies were done for research purposes only.

Formalin-fixed tissues were paraffin embedded, and 5-µm-thick sections were prepared, dewaxed in xylene, and rehydrated through graded concentrations of alcohol to water and then boiled for 10 minutes in sodium citrate buffer. Then, sections were incubated with blocking buffer for 1 hour at room temperature. Antihuman Sema3E antibody (R&D Systems, Minneapolis, Minn) or isotype control IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa) was added, and sections were incubated overnight at 4°C. Slides were then washed twice with TRIS-buffered saline followed by incubation for 1 hour at room temperature with biotin-conjugated secondary antibodies. Slides were washed extensively with TRIS-buffered saline and incubated with streptavidin-alkaline phosphatase for 30 minutes at room temperature afterward. Finally, slides were developed using Fast Red (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) and counterstained with modified Mayer's hematoxylin (Fisher Scientific, Fair Lawn, NJ). Mounted slides were visualized by Axioskop 2 mot plus microscope using AxioVision software (Carl Zeiss, Inc, Thornwood, NY).

Sema3E level was determined in human BALF and also supernatants of bronchial epithelial cell cultures from patients with asthma and healthy donors by using an ELISA kit according to the manufacturer's instructions (R&D Systems). Samples were concentrated 10-fold by centrifugation using 10K cutoff concentrating centrifugal filter units Ultracel from Milipore before performing ELISA.

Epithelial cells were isolated from bronchial biopsy specimens of patients with asthma and healthy individuals and confirmed by immunofluorescence and flow cytometry as we described previously.¹¹ Total mRNA and supernatant samples were obtained from cultured airway epithelial cells from patients with asthma and healthy controls and kept at -80°C until they were used to assess Sema3E expression at message and protein levels, respectively. Total mRNA was extracted from the cultured human bronchial airway epithelial cells (passages 2-5) by using TRIzol (Life Technologies, Burlington, Ontario, Canada). RNA (2 µg) was used as a template to synthesize cDNA by MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, Calif). Relative expression of the SEMA3E and GAPDH genes was measured by quantitative real-time analysis. The forward and reverse-specific primer sequences, the size of the amplified fragment, and the annealing temperature for SEMA3E were as follows: Forward: 5'-AAAG CATCCCAACAACTG-3' Reverse: 5'-CTGGCTCGAGAC CCTTACTG-3', 155 bp, 58°C.

GraphPad Prism 5.0 software was used for statistical analysis and values were presented as the mean ± SEM. Data were analyzed by unpaired *t* test or 1-way ANOVA followed by the Bonferroni multiple comparisons post hoc test. Statistical correlation between Sema3E expression and FEV₁% was examined by performing Pearson R test.

We thank the staff at the Respiratory Health Network Tissue Bank for their valuable assistance. Specimens of bronchial biopsies were taken from the IUCPQ site of the Respiratory Health Network Tissue Bank of the FRQS.

Hesam Movassagh, PhD^a

Lianyu Shan, MSc^a

Jamila Chakir, PhD^b

John F. McConville, MD^c

Andrew J. Halayko, PhD^{d,e}

Latifa Koussih, PhD^a

Abdelilah S. Gounni, PhD^a

From ^athe Department of Immunology, Rady Faculty of Health Sciences, Max Rady College of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; ^bCentre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie du Quebec, Université Laval, Quebec City, Quebec, Canada; ^cthe Department of Medicine, University of Chicago, Chicago, Ill; ^dthe Department of Physiology and Pathophysiology, Rady Faculty of Health Sciences, Max Rady College of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; and ^eBiology of Breathing Theme, Children's Hospital Research Institute of Manitoba, Winnipeg, Manitoba, Canada. E-mail: abdel.gounni@umanitoba.ca.

This work was supported by the Canadian Institutes of Health Research (MOP grant no. 115115), the National Sciences and Engineering Research Council (grant no. RG PIN/386289), and the Children Hospital Research Foundation (grant no. 115115 to A.S.G.). H.M. was supported by Research Manitoba-Children's Hospital Research Institute of Manitoba Studentship.

Disclosure of potential conflict of interest: H. Movassagh has a patent pending through the University of Manitoba. J. F. McConville has provided expert testimony on behalf of the US government (Domino vs. United States, 15 C 5579); has received grants from the US Department of Health and Human Services, Health Resources and Services Administration (grant no. 1 TOBHP285660100); and has received payment for lectures from the American College of Chest Physicians. A. S. Gounni has received a grant from the Canadian Institutes of Health Research and has a patent pending through the University of Manitoba. The rest of the authors declare that they no relevant conflicts of interest.

REFERENCES

- Hekking PP, Bel EH. Developing and emerging clinical asthma phenotypes. *J Allergy Clin Immunol Pract* 2014;2:671-80, quiz 81.
- Kumanogoh A, Kikutani H. Immunological functions of the neuropilins and plexins as receptors for semaphorins. *Nat Rev Immunol* 2013;13:802-14.
- Shim EJ, Chun E, Kang HR, Cho SH, Min KU, Park HW. Expression of semaphorin 3A and neuropilin 1 in asthma. *J Korean Med Sci* 2013;28:1435-42.
- Nkyimbeng-Takwi EH, Shanks K, Smith E, Iyer A, Lipsky MM, Detolla LJ, et al. Neuroimmune semaphorin 4A downregulates the severity of allergic response. *Mucosal Immunol* 2012;5:409-19.
- Mizutani N, Nabe T, Yoshino S. Semaphorin 7A plays a critical role in IgE-mediated airway inflammation in mice. *Eur J Pharmacol* 2015;764:149-56.
- Movassagh H, Shan L, Halayko AJ, Roth M, Tamm M, Chakir J, et al. Neuronal chemorepellent Semaphorin 3E inhibits human airway smooth muscle cell proliferation and migration. *J Allergy Clin Immunol* 2014;133:560-7.
- Bartemes KR, Kita H. Dynamic role of epithelium-derived cytokines in asthma. *Clin Immunol* 2012;143:222-35.
- Movassagh H, Saati A, Nandagopal S, Mohammed A, Tatari N, Shan L, et al. Chemorepellent semaphorin 3E negatively regulates neutrophil migration in vitro and in vivo. *J Immunol* 2017;198:1023-33.
- Movassagh H, Shan L, Mohammed A, Halayko AJ, Gounni AS. Semaphorin 3E deficiency exacerbates airway inflammation, hyperresponsiveness, and remodeling in a mouse model of allergic asthma. *J Immunol* 2017;198:1805-14.
- Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 2000;162:2341-51.
- Chakir J, Dube J, Laviolette M, Goulet F, Germain L, Auger F, et al. Isolation and characterization of human airway fibroblasts in culture. *Methods Mol Med* 2000;44:53-65.

Table E1: Clinical characteristics of asthmatic and healthy individuals subjected to bronchoscopy

Asthma severity	Gender	Age	Smoking status	Allergy	Medication	Dose (µg/day)	PC20 (mg/ml)	FEV1 (%)
Mild #1	F	19	No	Yes	Terbutaline	PRN	0.41	93
Mild #2	F	24	No	Yes	Salbutamol Antihistamine	PRN PRN	2.93	104
Mild #3	M	29	No	Yes	Salbutamol	PRN	0.04	88
Mild #4	M	33	Ex	Yes	Salbutamol	200	1	88
Mild #5	F	28	Ex	Yes	Salbutamol	PRN	0.71	116
Mild #6	F	19	No	Yes	Salbutamol	PRN	3.70	111
Mild #7	F	55	No	Yes	Salbutamol	PRN	0.26	71
Moderate #1	F	21	No	Yes	Salbutamol	PRN	0.06	118
Moderate #2	F	20	Ex	Yes	Formoterol Salbutamol Fluticasone	24 PRN 250	0.16	104
Moderate #3	F	21	No	Yes	Terbutaline	PRN	0.16	83
Moderate #4	M	32	No	Yes	Terbutaline Beclomethasone	PRN 100	3.27	58
Moderate #5	F	26	No	Yes	Salbutamol	PRN	2.08	100
Moderate #6	F	25	No	Yes	Fluticasone	1000	0.85	101
Moderate #7	F	25	No	Yes	Formoterol Terbutaline Fluticasone	PRN 500 625	0.44	93
Severe #1	M	20	Ex	Yes	Montelukast Salbutamol Salmeterol- Fluticasone	10 PRN 500- 2 puffs	0.16	65
Severe #2	F	21	Ex	Yes	Terbutaline Budesonide	PRN 1200	2.7	103
Severe #3	F	65	Ex	Yes	Salmeterol, Salbutamol Beclomethasone	50 PRN 600	0.11	77
Severe #4	F	23	No	Yes	Fluticasone	1000	0.17	98
Severe #5	F	38	No	Yes	Salmeterol Salbutamol Fluticasone	100 PRN 1000	0.81	93
Severe #6	F	45	No	Yes	Salbutamol Terbutaline Budesonide	PRN PRN 1600	0.58	105
Severe #7	F	21	Ex	Yes	Terbutaline, Budesonide	PRN 1200	0.16	103
Severe #8	F	52	No	Yes	Salbutamol, Fluticasone	400 1000	2.7	54

Severe #9	M	58	No	Yes	Salbutamol or Terbutaline Symbicort Beclomethasone Montelukast Prednisone	PRN 1200 1200 10 15	ND	54
Severe #10	M	35	No	Yes	Salbutamol or Terbutaline Symbicort Montelukast Prednisone	PRN 1200 10 7.5	ND	68
Severe #11	M	67	Ex	Yes	Salbutamol or Terbutaline Fluticasone or Salmeterol Ciclesonide	PRN 1000 1000	ND	89
Severe #12	M	69	Ex	Yes	Symbicort Ciclesonide	800 1600	ND	46
Healthy #1	M	23	Ex	No			50.88	91
Healthy #2	M	20	No	No			53.31	97
Healthy #3	F	31	No	No			169.6	107
Healthy #4	M	19	Ex	No			>128	106
Healthy #5	F	23	No	No			219.45	111
Healthy #6	M	27	No	No			>64	100
Healthy #7	F	27	No	No			>128	105
Healthy #8	M	31	No	No			>128	108

FEV1: Forced Expiratory Volume in 1 second

ND: Not Determined

PC20: Provocative concentration of methacholine causing a 20% fall in FEV1

PRN: Per Need

Table E2: Clinical profile of asthmatic and healthy subjects used in human BALF studies

Subjects	Gender	FEV1 % pred	Steroid1	Steroid2	Steroid3	Other asthma medicati ons	Asthma duration (yr)	Total IgE	Allergens (serum)
Severe #1	F	50	Advair 500/50	OCS		Singulair , Albuterol	24	1131	Cat, Dog, Aspergillus, Candida, <i>A. alternata</i> , <i>S. rostrata</i> , Ragweed
Severe #2	F	38	Advair 500/50	OCS	Budesonide	Combive nt	57	368	Cat, Aspergillus, HDM (med+)
Severe #3	M	42	Advair 500/50			Singulair , Albuterol	46	66.9	Cat, Dog
Severe #4	F	25	Advair 250/50			Singulair , Spiriva, Albuterol	72	299	<i>S. herbarum</i> , <i>P. betae</i> , <i>E. purpurascens</i> , <i>T. rubrum</i> , <i>B. cinerea</i> , <i>S. rostrata</i> , <i>F. proliferatum</i>
Severe #5	F	50	Advair 250/50			Albuterol	7	881	Negative
Severe #6	F	59	Symbicort 160/4.5			Singulair , Albuterol	70	25.7	Negative
Severe #7	F	41	Fluticasone 220			Salmeter ol 50, Maxair	14	87	Negative
Healthy#1	M	95							
Healthy#2	F	102							
Healthy#3	F	96							
Healthy#4	M	69							
Healthy#5	F	92							
Healthy#6	F	93							
Healthy#7	M	72							

FEV1: Forced Expiratory Volume in 1 second

OCS: Oral Corticosteroids