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BRONCHIAL INFLAMMATION AND HYPERRESPONSIVENESS IN WELL-CONTROLLED ASTHMA

Running head: Inflammation in well-controlled asthma

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ABSTRACT

Background: Little research has been devoted to the characteristics of bronchial inflammation in patients with stable, well-controlled asthma.

Objective: The aim of this study was to assess the degree and type of airway inflammation and investigate the relationship between inflammation and bronchial hyperresponsiveness in patients with well controlled asthma.

Methods: A cross-sectional study was conducted in 84 adult patients (43 men, mean age 43 years) with documented well-controlled asthma. Induced sputum samples were obtained and cell types determined by differential cell count. Spirometry and methacholine challenge testing were performed. Asthma Control Questionnaire (ACQ) was used to assess symptoms. Patients were included if their ACQ score was < 0.75.

Results: Fifty-nine patients had persistent bronchial inflammation: 28 cases were considered eosinophilic, 28 neutrophilic, and 3 mixed. Median (range) percentage of eosinophils was 4% (0-64) in patients testing positive to methacholine challenge (n=66) and 1% (0-3) in those testing negative (n=18) ($p=0.003$). A positive correlation was found between eosinophil percentage and the methacholine dose/response ratio ($r=0.477$, $p=0.0001$). The geometric mean (95% CI) of the methacholine PC20 was 1.74 mg/mL (1.04-2.93) in patients with eosinophilic inflammation and 4.14 mg/mL (2.5-6.84) in neutrophilic inflammation ($p=0.03$).

Conclusions: Inflammation and bronchial hyperresponsiveness persist in most patients with well-controlled asthma.

Clinical relevance: The study demonstrates that eosinophilic or neutrophilic inflammation persisted in most well-controlled asthma patients despite the fact that their condition was controlled and therefore measurement of bronchial inflammation seems essential to achieve proper asthma control.

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways in which many cell and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing particulate at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment [1]. The asthma definition includes four domains: symptoms and variable airway obstruction, which are readily amenable to assessment by clinicians, and airway inflammation and airway hyperresponsiveness, which characterize the underlying disease process, but are less accessible in clinical practice. The classic system for grading asthma severity was established from the first two domains, and specific treatment was recommended based on the severity of the condition [1]. Nonetheless, this approach has been gradually replaced by the idea of judging severity based on the need for treatment to achieve asthma control [2]; that is, the extent to which the manifestations of asthma are reduced or eliminated by treatment. Assessment of asthma control should incorporate two components: current clinical control (symptoms, use of medication to relieve symptoms, and clinical lung function) and future risk (exacerbations and rapid decline in lung function) [3].

For asthma to be considered well-controlled there should be a decrease in the number of exacerbations, and recent studies have shown that this can be more easily achieved when treatment is adjusted according to the degree of bronchial inflammation measured by eosinophil count in induced sputum than by adjusting based on clinical parameters [4-5]. Although recommendations from the Cochrane Library [6] advocate this approach, there remain several uncertainties. Recent studies have suggested that there are different cellular phenotypes in bronchial asthma [7], and that the importance of eosinophils is questionable in some of them [8]. Furthermore, sputum induction is not successful in all patients, the sample obtained may not be adequate, and the analysis requires immediate processing of the sample, which means that a properly equipped laboratory must be available in the center where induction is performed, a condition that is not met in all hospitals (9). Thus, several strategies for indirect evaluation of

inflammation have been proposed to guide asthma treatment, such as measurement of exhaled nitric oxide [10], study of pH in exhaled breath condensate (EBC) [11], and determination of airway hyperresponsiveness [12].

In this scenario, in which measurement of bronchial inflammation seems essential to achieve proper asthma control, only a few studies have indirectly examined the characteristics of airway inflammation in patients with well-controlled asthma [12-14], and to our knowledge, only two studies have directly focused on evaluating markers of airway inflammation in stable, well controlled asthma [15-16]. The aim of this study is to determine the type and degree of bronchial inflammation present in patients with well-controlled asthma, and the relationship between the inflammation present and the degree of bronchial hyperresponsiveness. Moreover, to our knowledge, this is the first study that uses the asthma control questionnaire (ACQ) to define asthma control when studying well-controlled asthmatics.

MATERIAL AND METHODS

Patients and design

This is a cross-sectional study, approved by the Ethics Committee of our tertiary hospital. All patients provided written informed consent for participation in the study. Patients were selected from the database of a specialized unit for bronchial asthma (Figure 1). All patients had shown reversible airway obstruction on methacholine challenge testing or bronchodilator testing, or variability >20% in the peak flow recording.

The study included 84 patients with mild (n=55) or moderate (n=29) asthma according to the Global Initiative for Asthma (GINA) classification (2), and no other lung diseases. The following inclusion criteria were applied: age 18 to 65 years, at least 1 year since the asthma diagnosis, controlled asthma established by a score lower than 0.75 on the ACQ [17] and no exacerbations defined according to GINA criteria [2] over the previous year, FEV1 >70% the reference value, and asthma control attained by the use of inhaled corticosteroids at doses of 200-800 µg/day (equivalent dose of budesonide) with or without addition of a long-acting beta

agonist (LABA) at a dose no greater than 100 µg/day. All patients had to have successfully undergone induced sputum collection.

Before their scheduled visit to the pulmonary function laboratory, patients were instructed to discontinue treatment with inhaled corticosteroids and LABA 24 hours previously and the use of short-acting beta agonists at least 6 hours previously. All patients first answered the ACQ to ensure that asthma was stable. Spirometry and methacholine challenge were performed, and sputum induction was carried out.

Atopy and smoking status

Patients were considered atopic if they had at least one positive prick test to any of the common environmental allergens [18]. Non-smokers were patients who had never smoked and ex-smokers were those who had not smoked for at least 6 months. The number of pack-years was calculated in all cases.

Spirometry and methacholine challenge

Spirometry was performed with a Datospir 200 (Sibel, Barcelona) instrument, according to European Respiratory Society (ERS) and American Thoracic Society (ATS) guidelines [19]. The reference values used were those proposed for the Mediterranean population [20]. Bronchial challenge with methacholine was undertaken following the method described by Chai et al [21]. Briefly, using a Mefar MB3 (Mefar, Ele H2O, Mediacalli, Brescia, Italy) dosimeter, increasing doses of methacholine (0.03 mg/mL-16 mg/mL) were inhaled at 3-minute intervals until FEV1 had fallen by 20% of the baseline value or the subject had inhaled the maximum concentration of methacholine. The methacholine concentration required to produce a 20% drop in FEV1 was designated PC20 and expressed in mg/mL. In keeping with the ATS guidelines [22], methacholine challenge was considered negative if the PC20 FEV1 was higher than 16 mg/mL. In all patients, the methacholine dose-response ratio (DRR) was calculated as the percentage fall in FEV1 at the last dose, divided by the total dose administered [15].

Sputum induction and processing

Sputum induction was performed by inhaling increasing concentrations of hypertonic saline (3%, 4%, and 5%) for 5 minutes each. The nebulized solution was generated by an OMRON ultrasonic nebulizer (OMRON EUROPE BV, Hoofddorp, The Netherlands) with an output of 1 mL/min. FEV1 was measured at the start and at completion of each inhalation period to ensure the patient's safety. Patients were asked to blow their nose, rinse their mouth, and swallow water to minimize contamination with post-nasal drip and saliva. They were instructed to cough into a sterile container, and the expectorate was processed within 2 hours. Sputum examination was performed as described by Pizzichini et al. [10].

In accordance with other authors [23-24], we considered that a patient had eosinophilic inflammation when induced sputum eosinophil count was >2%, neutrophilic inflammation when neutrophil count was >60% and mixed inflammation when eosinophil count was >2% and neutrophil count >60%. In cases showing an eosinophil count <2% and neutrophil count <60%, patients were considered to have paucigranulocytic inflammation.

Statistical analysis

Analyses of the methacholine PC20 and DRR were carried out on log-transformed data. Results are presented as the geometric mean (95% confidence interval [CI]) for PC20 and the DRR, and the median (range) for all other variables, unless otherwise stated. Differences between groups were analyzed using the Kruskal-Wallis test. Spearman's rank correlation test was applied to determine correlations between the various parameters studied. SPSS 11.0 for Windows (SPSS, INC, Chicago, IL) was used for the statistical analysis.

RESULTS

Of the original 147 patients assessed for eligibility (Figure 1), 84 were able to produce sputum, yielding a success rate of 69%. The baseline characteristics of the 84 asthma patients ultimately included in the study and the 46 who could not produce sputum are shown in Table 1. There were no significant differences in the baseline characteristics between the two groups of patients. The median (range) age of the 84 asthma patients studied was 41.5 (18 – 65) years and the median (range) duration of their condition was 6 (1 – 51) years. Eighteen patients tested negative on methacholine challenge. In patients testing positive, the mean duration of their condition was longer, and they had a higher percentage of eosinophils in sputum and lower FEV1 values than those testing negative (Table 2). Patients with a positive methacholine result and PC20 $\leq$ 4 mg/mL showed significantly lower FEV1 and FEV1/FVC% findings and a higher eosinophil count than those with a PC20 of >4 to 16 mg/mL (Table 2). A significant correlation was found between sputum eosinophil percentage and the DRR ($r=0.477$, $p<0.0001$) (Figure 2) or PC20 ($r=-0.517$, $p<0.0001$). Eighteen patients were smokers at the time of the study. There were no significant differences in the presence or type of inflammation observed (neutrophilic or eosinophilic) between smokers and non-smokers.

Persistent bronchial inflammation was documented in 59 patients, and was classified as eosinophilic in 28, neutrophilic in 28, and mixed in 3. The inflammation was considered paucigranulocytic in 25 patients. Results for the variables studied according to the type of inflammation (neutrophilic, eosinophilic, or paucigranulocytic) are shown in Table 3. Significantly lower PC20 values were only found in patients with a positive methacholine challenge and eosinophilic inflammation relative to those with a neutrophilic or paucigranulocytic type.

DISCUSSION

The most relevant findings in this study are the persistent bronchial inflammation seen in patients with asthma treated with inhaled corticosteroids and in a controlled phase of the disease, the high percentage of patients with neutrophilic inflammation, and the presence of a greater degree of direct airway hyperresponsiveness in patients with eosinophilic asthma. Although similar findings have been found in patients with asthma during the diagnostic process, this is the first reported data in patients with well-controlled asthma after years of disease evolution.

Perhaps one the most important advances in the treatment of asthma occurred when the inflammatory component of the condition was demonstrated and found to be satisfactorily treated with inhaled corticosteroids. Early pathologic studies in patients with mild asthma who were not treated with corticosteroids reported high eosinophil and lymphocyte counts in the mucosa of the large airways. The number of these cells decreased significantly and overall lung function improved following high doses of inhaled corticosteroids [25-26]. Later studies showed that better asthma control is obtained when treatment is adjusted to the number of eosinophils observed in samples of induced sputum, in conjunction with the general recommendations based on clinical and spirometric data [4-5]. Although a recent Cochrane review has established this as a demonstrated fact [6], this approach can be difficult to apply in daily practice, and this may be explain, in part, the findings of the present study.

Treatment adjustment and asthma control in our study were carried out in accordance with the GINA strategy, which is essentially based on clinical factors [2]. Therefore, it is not surprising that bronchial inflammation was detected in 66% of the cross-sectional population studied, despite the fact that their condition could be considered well controlled from the clinical viewpoint (ACQ <0.75 and no exacerbations in the previous year). Similar findings have been reported by other authors. In a population of 76 adult asthma patients with total asthma control based on GINA/NIH guidelines, Hanxiang et al [16] observed higher levels of eosinophils, eosinophil cationic protein and IL-5 in comparison with healthy subjects, suggesting that airway

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inflammation persisted during total asthma control. Madhuban et al [27], reported similar results in a pediatric population with well-controlled asthma and presenting exercise-induced bronchoconstriction. Finally, Leuppi et al [15] found that airway hyperresponsiveness in 31 clinically well-controlled asthmatics appeared to be independent of eosinophilic airway inflammation. Although Madhuban et al [27], establish asthma control from ACQ, to our knowledge, this is the first study investigating the relationship between asthma control according to ACQ and objective markers of airway inflammation in an adult population. Therefore, probably there is a need for studies investigating this relationship.

What is more uncertain is the significance of the persistent inflammation in these patients. Our study patients had been asymptomatic over the previous year and were using low or moderate doses of inhaled corticosteroids. It could be expected that if treatment had been adjusted based on the degree of inflammation, higher doses of corticosteroids would be administered and the patient would therefore be classified as having severe disease; however, the practical benefits would not have been greater than those observed, at least in terms of immediate control of the condition. Our study design does not enable future risk to be established in these patients. Thus, we cannot know whether patients who present greater bronchial inflammation despite their current disease control will have a greater risk of experiencing exacerbations or deteriorated lung function later in life [3].

Another interesting finding from the present study was the fact that 33% of patients presented neutrophilic asthma. At least three pathological phenotypes of asthma have been proposed based on the predominant cell type involved: eosinophilic, neutrophilic, and paucigranulocytic [7]. Several authors [24, 28-29] have suggested that many patients with neutrophilic inflammation can have concomitant eosinophilic inflammation, but this was not the case of our series, in which only 3 of the patients included showed mixed inflammation. Although a role for neutrophilic inflammation has been established in severe asthma [30] and asthma exacerbations [31], neutrophils are not always considered important in stable asthma. Our findings are not consistent with this idea, and other studies in which neutrophils were found to

be the dominant inflammatory cell in sputum of subjects with mild to moderate asthma [29, 32] support our results.

It is possible that the neutrophilia found in some of our patients could be related to their smoking habit. Smoking is surprisingly common in asthma patients, with a prevalence relatively close to that of the general population [33-34], and a recent study has shown that the induced sputum neutrophil count in asthma patients who smoke is higher than in those who do not [35]. Moreover the levels of sputum eosinophil counts are reduced in smokers probably because the exogenous nitric oxide in cigarette smoke increasing the apoptosis of activated eosinophils [36]. Nonetheless, only 18 patients in our population were smoking at the time of the study and there were no significant differences in this group regarding the neutrophilic or eosinophilic status of inflammation. Some authors have hypothesized that smokers with neutrophilic asthma could have a low response to corticoid treatment; thus, smoking would be a risk factor for severe asthma [37]. The present study was not designed to respond to this question because our patients all had well-controlled asthma (ACQ <0.75). We can affirm, however, that it is possible to achieve disease control in at least some patients with mild or moderate asthma even though they may be smokers and present neutrophilic bronchial inflammation.

Other factors with a possible influence on neutrophilia include corticosteroid treatment exposure to air pollution, and the presence of airway infections [7]. All patients were taking corticosteroids, however, at least over the previous year. This fact could have an effect on neutrophilia in two ways, by sustaining the life of neutrophils or by eliminating eosinophils in patients who initially had mixed inflammation. In this sense Cowan DC et al, assessing the inflammatory cell phenotypes in asthma after eliminating potentially confounding effects found that corticosteroid treatment was associated with increased airway neutrophils and a switch to a neutrophilic phenotype in some patients [38]. Our study design did not enable assessment of the effect of pollution, and infections are unlikely because all patients were stable during the study and none had experienced exacerbations in the previous year as a requirement for inclusion. Although exacerbation is not synonymous with infection, we believe it is unlikely that infection had an influence on the neutrophilia observed.

Similar to induced sputum eosinophil count, adjusting asthma treatment according to bronchial hyperresponsiveness measurement has been proposed as a better method for achieving asthma control than the use of conventional guidelines [39]. However, in this case, control is attained with administration of high doses of inhaled corticosteroids, without significant changes in the degree of bronchial hyperresponsiveness. These findings support the notion that several factors can contribute to direct bronchial hyperresponsiveness, and these can be broadly grouped into two categories: persistent and variable [40]. The persistent contribution to bronchial hyperresponsiveness has been attributed to structural changes in the airway, such as subendothelial thickening, subbasement membrane thickening, smooth muscle hypertrophy, matrix deposition, and altered vascular components. The variable aspect is believed to relate to inflammatory airway events, which vary and are influenced by infection and environmental factors such as pollution.

The persistent factors are likely those that best explain the enduring bronchial hyperresponsiveness in the present study. bronchial hyperresponsiveness to methacholine was present in most patients, even though they had been under lengthy treatment with inhaled corticosteroids. The fact that all the patients had well-controlled asthma and were stable for at least one year prior to inclusion upholds the idea that therapy with inhaled corticosteroids and occasional LABA use can control the variable factors, whereas the persistent factors remain and perpetuate bronchial hyperresponsiveness. In this regard, it is important to note that patients testing positive on methacholine challenge had a longer history of asthma and a lower FEV1, with no differences in age, compared to patients testing negative. Similar findings were obtained by McGrath KW et al in a sample of over 300 subjects who report a history of asthma. The authors found that 27% had a negative methacholine challenge test. Compared to the methacholine positive subgroup, the clinical characteristics of the methacholine negative subgroup included an older age of asthma onset and better lung function [41].

It is also interesting that the degree of direct bronchial hyperresponsiveness was much higher in patients with eosinophilic inflammation. This finding suggests that when direct bronchial

hyperresponsiveness persists despite inhaled corticosteroids, it will be more severe in patients with eosinophilic inflammation than in those with neutrophilic inflammation or normal induced sputum eosinophil and neutrophil counts. It has been reported that patients with severe corticosteroid-dependent asthma showing eosinophilic inflammation required more intubations than those with other types of inflammation (42). This could be because eosinophilic inflammation might cause greater changes in the airway architecture (eg, subendothelial thickening, subbasement membrane thickening, smooth muscle hypertrophy), which would contribute to perpetuating bronchial hyperresponsiveness, although this hypothesis remains to be demonstrated.

Some limitations of the present study should be mentioned. First, it was assumed that patients adhered to treatment. We cannot exclude the possibility that treatment compliance was not optimal in some patients because of the absence of symptoms, and that this may have been the cause of eosinophilia and bronchial hyperresponsiveness in some cases. Second, the inclusion of smokers could be the cause of the neutrophilia found in some of our patients and even may cause decreased levels of eosinophils. Lastly, it should be noted that the ACQ was designed for patient follow-up, whereas in this study it was used at one time point to reinforce the clinical evidence that the patient's condition was controlled.

In conclusion, eosinophilic or neutrophilic inflammation persisted in most well-controlled asthma patients despite the fact that their condition was controlled. Direct airway hyperresponsiveness was documented in nearly all the patients and was higher in those with eosinophilic inflammation.

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Conflict of interest: The authors declare no conflicts of interest.

REFERENCES

1. Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. NIH publication number 95-3659^a. Bethesda, National Institutes of health, 1995.
2. Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention 2010. Available from www.ginasthma.com
3. Taylor DR, Bateman ED, Boulet LP, Boushey HA, Busse WW, Casale TB, Chanez P, Enright PL, Gibson PG, de Jongste JC, Kerstjens HA, Lazarus SC, Levy ML, O'Byrne PM, Partridge MR, Pavord ID, Sears MR, Sterk PJ, Stoloff SW, Szeffler SJ, Sullivan SD, Thomas MD, Wenzel SE, Reddel HK. A new perspective on concepts of asthma severity and control. *Eur Respir J* 2008;32:545-554.
4. Green RH, Brightling E, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw J, Pavord ID. Asthma exacerbations and sputum eosinophil counts; a randomised trial. *Lancet* 2002;360:1715-21.
5. Jayaram L, Pizzichini MM, Cook RJ, Boulet LP, Lemièrre C, Pizzichini E, Cartier A, Hussak P, Goldsmith CH, Laviolette M, Parameswaran K, Hargreave FE. Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. *Eur Respir J* 2006;27:483-494.
6. Petsky HL, Kynaston JA, Turner C, Li A, Cates CJ, Lasserson TJ, Chang AB. Tailored interventions based on sputum eosinophils versus clinical symptoms for asthma in children and adults (review). *The Cochrane Library* 2009, Issue 1. Available from www.thecochranelibrary.com.
7. Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet* 2006; 368:804-13.
8. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 2002;57:643-648.
9. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, Gleich GJ, Dolovich J, Hargreave FE. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308-317.

- Accepted Article
10. Smith AD, Cowan JO, Brasstt KP, Herbison GP, Taylor DR. Use of exhaled nitric oxide measurements to guide treatment in chronic asthma. *N Engl J Med* 2005;353:2163-2173.
 11. Kostikas K, Papatheodorou G, Gans K, et al. PH in expired breath condensate in patients with inflammatory airway diseases. *Am J respir crit care med* 2002;165:1364-1370.
 12. Sont JK, Willems LN, Bel EH, van Kricken 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;159:1043-1051.
 13. Fuhlbrigge AL. Asthma severity and asthma control: symptoms, pulmonary function and inflammatory markers. *Curr Opin Pulm Med* 2004; 10: 1-6..
 14. Prosperini G, Rajakulasingam K, Cacciola R, Spicuzza L, Rorke S, Holgate ST, Di Maria G, Polosa R. Changes in sputum counts and airway hyperresponsiveness after budesonide: Monitoring anti-inflammatory response on the basis of surrogate markers of airway inflammation. *J Allergy Clin Immunol* 2002; 110: 855-61.
 15. Leuppi JD, Salome CM, Jenkins CR, Koskela H, Brannan JD, Anderson SD, Andersson M, Chan HK, Woolcock AJ. Markers of airway inflammation and airway hyperresponseveness in patients with well-controlled asthma. *Eur Respir J* 2001; 18: 444-450.
 16. Hanxiang N, Jiong Y, Yanwei C, Dunshuang W, Xuhong D, Xiaojun W, Changsheng L. Persistent airway inflammation and bronchial hyperresponsiveness in patients with totally controlled asthma. *Int J Clin Pract.* 2008 Apr;62(4):599-605.
 17. Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999;14:902-907.
 18. Pepys J. Atopy. In : Gell PGH, Coombes RRA, eds. *Clinical aspects of immunology*. Oxford Blackwell Scientific Publications, 1975.
 19. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J; ATS/ERS Task Force. Standardisation of spirometry. *Eur Respir J* 2005; 26(2):319-38
 20. Roca J, Sanchis J, Agusti-Vidal A, Segarra F, Navajas D, Rodriguez-Roisin R, Casan P, Sans S. Spirometric references values from a Mediterranean population. *Bull Eur Physiophatol Respir* 1986;22:217-2.

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21. Chai H, Farr RS, Froehlich LA, Mathison DA, McLean JA, Rosenthal RR, Sheffer AL, Spector SL, Townley RG. Standardization of bronchial inhalation challenge procedures. *J Allergy Clin Immunol* 1975;56:323-327.
 22. Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, MacIntyre NR, McKay RT, Wanger JS, Anderson SD, Cockcroft DW, Fish JE, Sterk PJ. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med*. 2000 Jan;161(1):309-29.
 23. Simpson JL, Scott RJ, Boyle MJ, Gibson PG. Differential proteolytic enzyme activity in eosinophilic and neutrophilic asthma. *Am J Respir Crit care Med* 2005;172:559-565.
 24. Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, Peters SP, Bleecker ER. Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. *J Allergy Clin Immunol* 2010;125:1028-1036.
 25. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, Holgate ST. Effect of an inhaled corticosteroid on airways inflammation and symptoms in asthma. *Am Rev respir Dis* 1992;256:669-674.
 26. Laitinen LA, Laitinen A, Haahtela T. A comparative study of the effects of an inhaled corticosteroid, budesonide, and a beta 2-agonist, terbutaline, on airways inflammation in newly diagnosed asthma: a randomized, double blind, parallel-group controlled trial. *J Allergy Clin Immunol* 1992;90:32-42.
 27. Madhuban AA, Driessen JM, Brusse-Keizer MG, van Aalderen WM, de Jongh FH, Thio BJ. Association of the asthma control questionnaire with exercise-induced bronchoconstriction. *J Asthma*. 2011 Apr;48(3):275-8.
 28. Wenzel SE, Schwartz LB, LangmackEL, Halliday JL, Trudeau JB, Gibbs RL, Chu HW. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit care med* 1999;160:1001-1008.
 29. Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001;119:1329-1336.

- Accepted Article
30. Jatakanon A, Uasuf C, Maziak W, Lim SM, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999;160:1532-1539.
 31. Fahy J, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995;95:843-852.
 32. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002;57:875-879.
 33. Althuis M, Sexton M, Prybylski D. Cigarette smoking and asthma severity among adult asthmatics. *J asthma* 1999;36:257-264.
 34. Morell F, Genover T, Muñoz X, García-Aymerich J, Ferrer J, Cruz MJ. Rate and characteristics of asthma exacerbations: the ASMAB I study. *Arch Bronconeumol* 2008;44(6):303-11.
 35. Boulet LP, Lemièrre C, Archambault F, Carrier G, Descary MC, Deschesnes F. Smoking and asthma. Clinical and radiologic features, lung function, and airway inflammation. *Chest* 2006;129:661-668.
 36. Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. *Eur Respir J* 2004;24(5):822-33.
 37. Cox G, Whitehead L, Dolovich M, Jordana M, Gauldie J, Newhouse MT. A randomized controlled trial on the effect of inhaled corticosteroids on airways inflammation in adult cigarette smokers. *Chest* 1999;115:1271-1277.
 38. Cowan DC, Cowan JO, Palmay R, Williamson A, Taylor DR. Effects of steroid therapy on inflammatory cell subtypes in asthma. *Thorax* 2010;65(5):384-90.
 39. Brannan JD. Bronchial hyperresponsiveness in the assessment of asthma control: Airway hyperresponsiveness in asthma: its measurement and clinical significance. *Chest* 2010;138(2 Suppl):11S-17S.
 40. Busse WW. The relationship of airway hyperresponsiveness and airway inflammation. *Chest* 2010;138(suppl):4s-10s.
 41. McGrath KW, Fahy JV. Negative methacholine challenge tests in subjects who report physician-diagnosed asthma. *Clin Exp Allergy* 2011;41(1):46-51.

42. Sont JK, Han J, van Krieken JM, Evertse CE, Hooijer R, Willems LNA, Sterk PJ.

Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity in patients treated with inhaled steroids. *Thorax* 1996;51:496-502

Table 1. Baseline characteristics of patients from whom sputum was obtained for analysis of inflammatory cell numbers and patients who were unable to produce sputum

Patients without sputum n = 46	<i>p</i>	Patients with sputum n = 84
Sex, n of subjects Male/Female 19 / 27	0.323	43 / 41
Age, y 41.7 (20 – 65)	0.570	41.5 (18 - 65)
Positive atopic status, n (%) of subjects 26 (57%)	0.225	51 (60%)
Years of asthma, 7 (1 – 62)	0.125	6 (1 - 51)
Smoking habit, n (%) / mean (SD) pack-years Never smoker 28 (61%) Ex-smoker 8 (17%) / 12.07 (7.86) Smoker (32.49) 10 (22%) / 24.52 (17.37)	0.630 0.325 0.230	49 (58%) 17 (20%) / 12.92 (9.30) 18 (21%) / 24.18
Daily IC dose (µg budesonide equivalent) mean (range) 345 (200 - 800)	0.430	392 (200-800)
Use of LABA, n (%) subjects 16 (35%)	0.565	26 (30.5%)
Baseline FVC % predicted 92.4 (58 - 114)	0.467	90 (67 - 141)
Baseline FEV1% predicted 93.3 (70 - 125)	0.120	89.5 (70 - 137)
Baseline FEV1/FVC 79.9 (53 - 94)	0.265	78 (54 - 93)
Methacholine challenge: Positive: n (%) of subjects/ PC20, mg/mL 9.45) 36 (78%) / 8.5 (6.06 - 9.20) Negative: n (%) of subjects 10 (22%)	0.312 0.420	66 (79%) / 8.04 (6.63 - 18 (21%)
Methacholine DRR -----	-----	11.46 (5.99 - 16.93)
Sputum eosinophils, % -----	-----	1 (0-64)
Sputum neutrophils, % -----	-----	52 (1-98)
ACQ 0.52 (0 - 0.75)	0.215	0.45 (0 - 0.75)

Abbreviations: ACQ, asthma control questionnaire; DRR, dose/response ratio; IC: inhaled corticosteroid; LABA, long-acting beta agonist
Data are presented as the geometric mean (95% confidence interval) for methacholine PC20 and DRR, and the median (range) for the remaining variables, unless otherwise indicated.

Table 2. Analysis of the variables in relation to qualitative methacholine assessment

Methacholine				
Positive		Positive	Negative	<i>p</i>
PC20: 0 – 4 mg/mL		PC20 ≤16 mg/mL PC20 >4 – 16 mg/mL <i>p</i> n = 66	PC20 >16 mg/mL <i>p</i> n = 18	
n = 31	n = 35			
Sex, M/F				
Age, years		41.5 (20 - 65)	42.0 (18 - 64)	0.600
42 (20 - 65)	39 (25 - 62)	0.729		
Years of asthma		10.7 (1 - 51)	3.0 (1 - 20)	0.009
10 (1 - 51)	7 (2 - 35)	0.906		
FVC, % predicted		88.5 (67 - 141)	97.5 (77 - 112)	0.069
84 (74 - 141)	93 (67 - 118)	0.097		
FEV1, % predicted		88.0 (70 - 137)	99.5 (70 - 125)	0.014
85 (70 - 137)	94 (71 - 115)	0.002		
FEV1/FVC, %		77.0 (54 - 93)	80.0 (72 - 91)	0.074
74 (54 - 91)	79 (65 - 93)	0.025		
Sputum eosinophils, %		4.0 (0 - 64)	1.0 (0 - 3)	0.003
2 (0 - 64)	0 (0 - 21)	0.001		
Sputum neutrophils, %		50.0 (1 - 98)	58.0 (15 - 97)	0.091
42 (7 - 95)	53 (1 - 98)	0.515		
Smoking habit, n (%)				
Never smoker		38 (57%)	11 (61%)	0.715
13 (42%)	25 (71%)	0.047		
Ex-smoker		13 (20%)	4 (22%)	0.521
11 (35%)	2 (6%)	0.020		
Smoker		15 (23%)	3 (17%)	0.315
7 (23%)	8 (23%)	0.622		
Atopic status:				
Positive, n (%) of subjects		41 (63%)	10 (53%)	0.319
18 (58%)	23 (66%)	0.481		
Daily IC dose				
(µg budesonide equivalent)		400 (200 - 800)	300 (200 - 800)	0.283
400 (20 - 800)	400 (200 - 800)	0.244		
Use of LABA, n (%) subjects		22 (33.3%)	4 (22.2%)	0.263
9 (29.0%)	13 (37.1%)	0.804		

Abbreviations: IC: inhaled corticosteroid; LABA, long-acting beta agonist. Data are expressed as median (range) for all variables unless otherwise indicated

Table 3. Analysis of the variables studied according to the type of bronchial inflammation observed

	A	B	C	A-B / A-C / B-C
	Paucigranulocytic inflammation	Eosinophilic inflammation	Neutrophilic inflammation	
	n = 25	n = 28	n = 28	p
Age, years	46.5 (22 - 65)	40.0 (20 - 64)	39.0 (18 - 65)	0.07 / 0.14 / 0.66
Sex, Male/Female	11 / 14	15 / 13	17 / 11	0.49 / 0.54 / 0.67
Smoking habit Never smoker / Ex-smoker / Smoker	17 / 4 / 4	18 / 6 / 4	11 / 9 / 8	0.84 / 0.09 / 0.07
Years of asthma	8.0 (2 - 35)	5.0 (1 - 42)	6.0 (1 - 51)	0.55 / 0.64 / 0.73
Atopy, yes/no	13 / 12	21 / 7	16 / 12	0.08 / 0.18 / 0.13
Methacholine, + / -	18 / 7	24 / 4	22 / 6	0.22 / 0.47 / 0.45
PC20 *	5.78 (3.82 - 8.50)	1.74 (1.04 - 2.93)	4.14 (2.50 - 6.84)	0.001 / 0.004 / 0.03
FVC	89.0 (67 - 114)	91.5 (74 - 141)	90.0 (73 - 112)	0.4 / 0.74 / 0.66
FEV1	87.0 (71 - 112)	89.0 (70 - 137)	90.0 (70 - 125)	0.8 / 0.75 / 0.43
FEV/FVC%	80.0 (69 - 93)	75.5 (54 - 91)	77.0 (61 - 93)	0.1 / 0.23 / 0.55

Data are presented as geometric mean (95% confidence interval) for methacholine PC20; and median (range) for all other variables unless otherwise stated; * Only patients with PC20 ≤16 mg/mL (n=66)

LEGENDS

Figure 1. Number of patients who were screened and completed the study.

Figure 2. Relationship between methacholine dose/response ratio and percentage of eosinophils.



