ORIGINAL ARTICLE

NEUTROPHIL PREDOMINANCE IN INDUCED SPUTUM FROM ASTHMATIC PATIENTS. THERAPEUTIC IMPLICATIONS AND ROLE OF CLARA CELL 16-KD PROTEIN

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Abstract Eosinophil is considered to be a main protagonist in asthma; however, often discordances between clinical manifestations and response to treatment are observed. We aimed to determine the occurrence of neutrophil predominance in asthma and to identify its characteristics on the basis of clinical-functional features, induced sputum cellular pattern and soluble molecules, to guide the appropriated anti-inflammatory therapy. A total of 41 patients were included in randomized groups: 21-40 year-old, with stable mild-to-severe asthma, steroid-naïve and non-smokers. An induced sputum sample was obtained under basal conditions, a second one after treatment with budesonide (400 μg *b.i.d.*) or montelukast (10 mg/d) for six weeks, and a final one after a 4-week washout period. By cytospin we evaluated eosinophil (EP) or neutrophil predominance (NP), and in supernatant we determined LTE_4 and CC16. Peak expiratory flow variability (PEFV) was measured. A total of 23/41 patients corresponded to EP and 18/41 patients to NP. The PEFV was higher in EP than in NP. LTE_4 was higher with NP than with EP. No difference was found for CC16. Montelukast reduced the predominant cell in both subsets, whereas budesonide only reduced eosinophils in EP. Budesonide and montelukast reduced PEFV in EP but not in NP. Considering the total treated-samples in each subset, CC16 level increased significantly in EP. In conclusion: a NP subset of asthmatic patients was identified. These patients show a lower bronchial lability; the leukotriene pathway is involved which responds to anti-leukotriene treatment. This phenotype shows a poor recovery of CC16 level after treatment.

Key words: asthma, neutrophil, CC16, budesonide, montelukast

Resumen ***Predominio de neutrófilos en esputo inducido de asmáticos. Implicancias terapéuticas y rol de la proteína 16-kD de la célula clara.*** El eosinófilo es considerado la célula protagonista principal en el asma; sin embargo, a menudo se observan discordancias entre las manifestaciones clínicas y la respuesta de los pacientes al tratamiento. Nos propusimos determinar la ocurrencia de predominio de neutrófilos en el asma e identificar las características clínico-funcionales, el patrón celular y las moléculas solubles del esputo inducido, para guiar el tratamiento apropiado anti-inflamatorio. Se incluyeron 41 pacientes: 21 a 40 años de edad, con asma estable leve a grave, no tratados con esteroides tópicos ni sistémicos y no fumadores. Se obtuvo una muestra de esputo inducido en condiciones basales, una segunda muestra después del tratamiento al azar con budesonida (400 μg dos veces al día) o el montelukast (10 mg/d) durante seis semanas, y una final después de un período de lavado de 4 semanas. En el frotis por citocentrifugado se evaluó el predominio de eosinófilos (EP) o neutrófilos (NP), y en el sobrenadante se determinó LTE_4, y CC16. Se midió la variabilidad del flujo espiratorio máximo (PEFV). Un total de 23/41 pacientes correspondieron al EP y 18/41 pacientes con NP. El PEFV fue mayor en el EP que en NP. LTE_4 fue mayor en NP que en EP. No se encontraron diferencias de los niveles de CC16 en ambos grupos. Montelukast redujo la célula predominante en ambos subgrupos, mientras que budesonida sólo redujo los eosinófilos en EP. Tanto budesonida como montelukast redujeron PEFV en EP, pero no en NP. El nivel de CC16 aumentó significativamente en el EP luego del tratamiento antiinflamatorio. En conclusión: se identificó un subgrupo de asmáticos NP que presentan una menor labilidad bronquial, la vía de los leucotrienos parece estar involucrada y responde al tratamiento anti-leucotrienos. Este fenotipo muestra una escasa recuperación del nivel de CC16 posterior al tratamiento.

Palabras clave: asma, neutrófilos, CC16, budesonida, montelukast

Bronchial asthma, an increasingly prevalent disease worldwide, is characterized by activation of inflamma-

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tory mediators and local infiltration of inflammatory cells, such as the eosinophil which is considered to be a main protagonist in asthma pathophysiology. However, daily management of asthmatic patients reveals often discordance between the clinical manifestations and the treatment response. This fact appears to be associated with the existence of varying patterns of inflammatory cells and mediators that require a specific diagnosis and a differential pharmacological treatment.

There is accumulating evidence demonstrating that asthma also occurs with neutrophils as the major inflammatory cell. The occurrence of neutrophils in bronchial secretion has been associated mainly with severe asthma[15]; neutrophils have also been associated with less severe astha[6-9], but these reports did not clarify if neutrophils might actually be a main protagonist in asthma because a strict selection of steroid-naïve and non-smoking patients has not be taken into consideration in these studies. Previous evidences by Gabrijelcic et al[10] have shown that platelet-activating factor, a precursor of leukotriene, may be involved in the pathogenesis of bronchial asthma inducing the sequestration of neutrophils in the lung and increment of urine LTE_4 level as showed Echazarreta et al[11]. Therefore, cysteinyl leukotriene could be involved in the pathogenesis of neutrophilic asthma.

On the other hand, bronchiolar Clara cell of respiratory epithelium secrete 16-kD Clara cell protein (CC16) also name CC10, an inmunomodulatory protein[2] that has been demonstrated to inhibit neutrophils chemotaxis.

Decreased CC16 levels have been found in serum and bronchoalveolar fluid of patients with chronic obstructive pulmonary disease[13] however, the relationship between CC16 content and inflammatory cell pattern in asthma has not been evaluated.

The goal of this study was to determine the occurrence of neutrophil predominance in asthma and identified its characteristics on the basis of clinical-functional features, induced sputum cellular pattern and soluble molecules, to guide the appropriated anti-inflammatory therapy.

Material and Methods

We included male and non-pregnant female patients with mild to severe asthma from our first visit outpatient pneumology clinic and enrolled for this study. The project was approved by the Ethics Committees of the *Facultad de Ciencias Médicas*, *Universidad Nacional de Córdoba*. All patients received detailed information on the risks and potential benefits of this study and provided written consent.

The inclusion criteria were: patients, 21 to 40-year old, with stable, mild to severe asthma according to the definition and classification of severity of National Heart, Lung, and Blood Institute/World Health Organization Global Strategy for Asthma Management and Prevention[14]; absence of respiratory infections, exacerbation of asthma within the preceding 6 weeks, and no previous treatment with oral or inhaled steroids or leukotriene receptor antagonists; all them were non-smokers, with no systemic or cardiopulmonary disease other than asthma. Atopy was not an entry criteria.

This is a randomized, single-blind, and parallel study. On the first visit, medical history of the patient was recorded following a standard questionnaire, and major aeroallergen skin tests and lung function tests were performed.

On the next visit, a first sample of induced sputum was obtained; selected plug's sputum samples were processed according to a validated protocol15, and the cellular fraction was separated to analyze inflammatory cells, and supernatant was

stored at -70° C to further evaluate CC16 and LTE4. Patients with eosinophil/neutrophil (E/N) ratio $\geq$ 1.4, calculated using absolute sputum cell count and a percentage of eosinophils >3 were classified as eosinophil predominant (EP). To classify patients as neutrophils predominant (NP) two criteria were considered again: a E/N ratio $\leq$ 0.6, and a percentage of neutrophils $\geq$ 16 taking into account the percentage of neutrophils found in a previous study with a group of health voluntaries16-17 Patients with E/N ratio between 1.4 and 0.6 were not included in the study The patients were randomly assigned; following a computer-generated schedule, and received either 400 mg b.i.d budesonide inhaled dry powder (Neumotex 400® Phoenix Argentina) or 10 mg montelukast oral tablet (Singulair®, MSD) once a day at bedtime irrespective of food for 6 weeks.

After six weeks' treatment, second sputum was collected and analyzed, and after a four-week washout period, a third induced sputum was studied (Fig. 1). Along the study period, the patients were instructed to complete a daily self-report card.

Baseline spirometric pre-bronchodilator parameters were recorded from the best of three attempts using a Compact Vitalograph spirometer (Compact Vitalograph, Buckingham, U.K.) with measurement variations of less than 5% were considered acceptable. A baseline measurement of forced expiratory volume in one second (FEV1) was carried out according to American Thoracic Society criteria18. Spirometric measurements were performed before each sputum induction. A skin prick-test for the major aeroallergens was implemented in the first visit.

The patient daily self-report card included: A peak expiratory flow rate, a daytime symptom score (DSS) and the use of rescue beta-2 agonist (RS). The peak expiratory flow (PEF) was registered twice daily at 9:00 am and 6:00 pm. Then the PEFV was calculated from the difference between the highest and lowest daily PEF reading divided by the highest PEF value multiplied by 10019. Daytime symptom score including the daytime and night asthma symptom scale, using a range of response categories from 0 to 3: 0= null, 1= mild, 2= moderate, 3= severe, according to Santanello with modifications20. The average was calculated from the number of times the patient used an inhaled β2 agonist per day.

The concentration of CC16 was measured in sputum supernatants by enzyme-linked immunosorbent assay, employing a commercial kit (Diamed Eurogen, Belgium). The
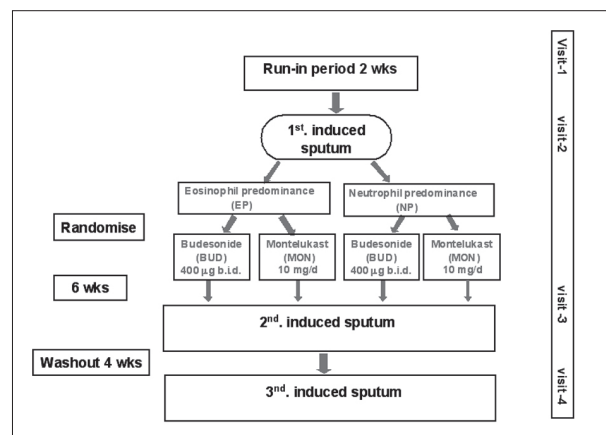
Fig. 1.– Study design. Definition of abbreviations: EP = eosinophil predominance; NP = neutrophil predominance; Wks = weeks; Bud = budesonide; Mon = montelukast.

minimum detectable concentration for this method is minor than 50 pg/ml.

A additional objective of this study was to determine whether asthma phenotype can be differentiated by the level of cysteinyl leukotrienes. The concentration of leukotriene in the sputum supernatants was quantified with an enzyme-linked immunosorbent assay applying a LTE4 commercial kit (Cayman Chemical, California).

Data were expressed as mean and 95% confidence intervals (CI). Descriptive statistics were used to summarize patient clinical and demographic characteristics. A generalized linear model with gamma distribution was used to analyse the two sputum cellular subsets. The group comparisons were conducted applying analysis of variance with Kruskal-Wallis test.

Wilcoxon rank tests analyses were used for between-group analyses at baseline and for the analysis of within-group treatment effect.

The appropriate sample size for both cells in each therapeutic arm was adequately powered (power 0.84%) to detect 0.30 as a minimum absolute change of eosinophil with the treatment, with a α of 0.05. The estimation was based on eosinophils because they exhibited higher variance than neutrophils. A $p < 0.05$ was considered significant.

Results

A total of 43 patients were examined and 41 patients meeting the strict inclusion criteria were enrolled in two groups according to the predominant cytological profile observed in the sputum. Two were excluded because had not criteria to classify.

A total of 23/41 patients (56%) exhibited eosinophil predominance in their sputum cytology; while in 18/41 (43%) patients the neutrophil was predominant cell. Both groups showed significant statistical differences when total eosinophil and neutrophil numbers were compared (Table 1).

The characteristics of the patients in both groups are summarized in Table 1. They show no difference in mean age and all patients demonstrated to be atopic. Although the FEV_1 values in EP and NP were not statistically different, the PEFV values were significantly higher in EP in comparison to the NP group (p = 0.03).

No significant difference was found in baseline level of CC16 between the two subsets.

Thirty three of the 41 patients that started the protocol completed the treatment period. Eight patients were excluded, 6 of these from the EP group: one because of travel, two because of pregnancy before randomization, one because of starting to smoke, and two because of the occurrence of a respiratory infection between the visits. Two subjects in the NP group did not complete the protocol because of travel.

TABLE 1.– *Patient characteristics, baseline lung function and cell countaccording to predominant cell*

Characteristics	EP	NP	p
Patients n	23 (56)	18 (43)	> 0.9
Sex M:F	11:12	13:5	0.45
Age yrs	26.18 (23-29)	29 (25-32)	0.16
GINA Asthma severity, n (%)			
Mild	8 (29)	9 (50)	
Moderate	10 (47)	6 (33)	
Severe	5 (24)	3 (17)	
Asthma duration yr	12 (8-14)	13 (6-20)	0.94
FEV1 % predicted	83 (74-91)	88 (74-101)	0.36
Mild	98 (83-113)	98 (76-121)	
Moderate	82 (70-93)	79 (81-101)	
Severe	62 /50-84)	49.5 (43-55)	
Pb FEV1 %	12 (8-14)	15 (3-27)	0.76
PEFV	32 (14-50)	14 (9-18)	0.03
Atopic n	23	18	0.99
DSS	(0.61-1.6)	0.97 (0.17-1.76)	0.43
RS	1 (0.5-1.4)	0.6 (0.1-2)	0.06
Eosinophils (%)	28 (15-41)	8.5 (3-14)	0.00
Neutrophils (%)	6 (1.5-1.0)	32 (18-46)	0.00

Data are presented as mean and 95% confidence intervals (CI). EP = eosinophil predominance. NP = neutrophil predominance. M = Male; F = Female; GINA = Global Initiative for Asthma; FEV1 = forced expiratory volume in one second; Pb = postbronchodilator; PEFV = peak expiratory flow variability; DSS = daytime symptoms score; RS = rescue beta-2 agonist.

TABLE 2.– *Clinical and functional characteristics of patients before and after budesonide and montelukast.*

A

Clinical Parameters	EP					
	Bl		Tto		Wsh	
	Bud	Mon	Bud	Mon	Bud	Mon
FEV_1 L	3.05	2.41	3.13	3.08	3.19	2.99
	(1.90-3.80)	(1.95-4.65)	(2.33-3.70)	(2.10-3.87)	(1.57-4.34)	(2.20-3.99)
FEV_1%	89	76	94	91	96	77
	(65-99)	(58-78)	(68-95)	(70-73)	(92-118)	(71-83)
PEFV	27	15	6*	9*	7*	8
	(7-100)	(13-50)	(2-7)	(5-16)	(4-8)	(4-17)
DSS	1.1	1	0.1†	0.5	0.1	1
	(0.1-2)	(0.2-3)	(0-0.8)	(0.2-4)	(0-2.5)	(0.2-3)
SR	0.6	1	1‡	0.2	0.1‡	0.3
	(0.1-2)	(0.1-2)	(2.3-3.7)	(0-3.2)	(0-4.7)	(0.03-3)

B

Clinical Parameters	NP					
	Bl		Tto		Wsh	
	Bud	Mon	Bud	Mon	Bud	Mon
FEV_1 L	2.76	3.09	2.92	3.25	3.30	3.26
	(1.30-4.31)	(2.34-4.59)	(2.04-4.33)	(2.46-4.59)	(1.09-4.26)	(2.14-5.34)
FEV_1%	98	98	112	112	112	112
	(81-102)	(68-102)	(87-113)	(87-113)	(68-114)	(68-114)
PEFV	11	14	25	10	21	13
	(4-16)	(8-26)	(10-26)	(5-37)	(21-34)	(8-30)
DSS	3.0	0.2	0.0 †	0.3	1.4	0.2
	(0.2-3)	(0.1-0.3)	(0-0.4)	(0-0.7)	(0-2)	(0-0.7)
SR	2.00	0.1	0.1*	0.02	0.40	0.07
	(0.1-2)	(0.1-0.3)	(0-0.2)	(0-0.6)	(0.1-1.9)	(0-0.8)

*A. EP: Eosinophil Predominance. B. NP: Neutrophil Predominance. Data are presented as mean with (95% CI). BL = baseline. Tto = treatment period; Wsh = washout period, PEFV = Peak expiratory flow rate variability; DSS = Daytime symptoms score; SR = short-acting ß agonist rescue. Bud = budesonide; Mon = montelukast; *:p = 0.0021 compared with BL; †: p = 0.0121 compared with BL; ‡: p = 0.0001, compared with BL.*

In the EP group, 17 were randomized and 9 placed in the budesonide arm and 8 in the montelukast. Eight out of 16 of the NP group were included to the budesonide arm and 8 to the montelukast. All basal subsets that emerged after randomization, demonstrated no statistical difference when comparing the different parameters here evaluated (not shown).

When analyzing functional respiratory parameters, budesonide and montelukast reduced the PEFV only in the EP group (p = 0.002). Budesonide but not montelukast improved significantly DSS in both EP and NP (p = 0.002) and RS in both EP (p = 0.0001) and in NP patients (p = 0.002) (Table 2 A-B).

Budesonide significantly reduced the numbers of eosinophils in EP (p = 0.01) and raised the neutrophils (p = 0.04). Montelukast significantly reduced eosinophils in EP (p = 0.04) and neutrophils in NP (p = 0.01) (Fig. 2).
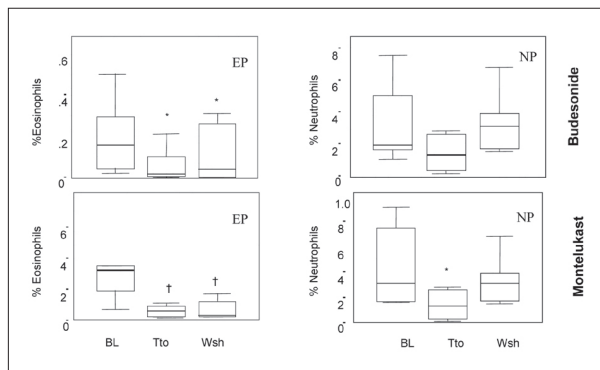
Fig. 2.– Budesonide and mMontelukast effects on cell percentage. Data are presented as mean and 95% CI. EP = eosinophil predominance; NP = neutrophil predominance; BL = baseline Tto = treatment; Wsh = washout; Bud = budesonide; Mon = montelukast. *: p = 0.01 compared with BL; †: p = 0.04 compared with BL.
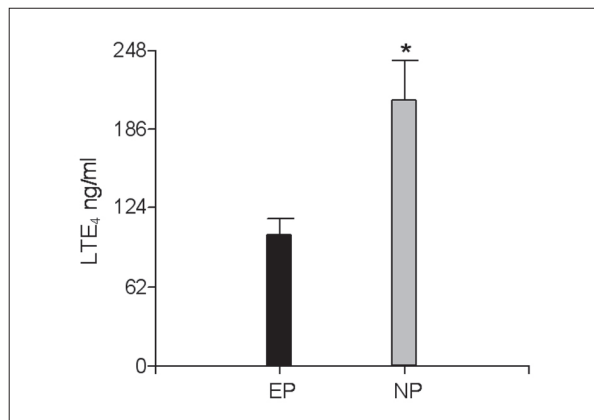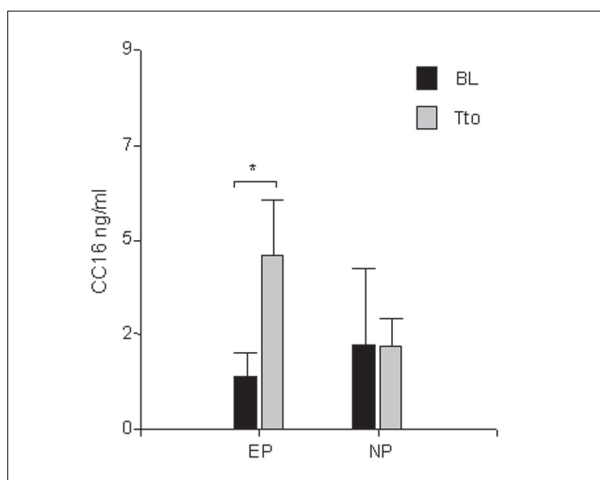

Fig. 3.– CC16 levels in induced sputum supernatant according to predominant cell subset before and after whole treatment. Data are presented as mean with 95% CI. EP = eosinophil predominance; NP = neutrophil predominance; BL = baseline; Tto = treatment period, *: p = 0.05.

Fig. 4.– Baseline levels of LTE4 of sputum supernatant according predominant cell subset. Values are presented as mean ± SEM. EP = eosinophil predominance. NP = neutrophil predominance *: p = 0.03.

Discussion

In the present study, a neutrophil-predominant subset of stable asthmatic patients was characterized and differentiated from the "classic" eosinophilic asthma. This group showed a lower bronchial lability than eosinophil-predominant group; it exhibited higher leukotriene levels and responded to leukotriene modifier treatment. Only eosinophils predominant group was able to increase epithelial CC16 anti-inflammatory protein after treatment.

Eosinophils in sputum represent a distinctive feature of asthma; however, eosinophilic inflammation alone does not explain the heterogeneity of the disease observed in the daily management of asthmatic patients[21], suggesting the existence of alternative inflammatory patterns involving other cell types and chemical mediators as major protagonists. Therefore, their specific diagnoses appear to be critical for a differential pharmacological treatment. A main contribution of the present report is the finding of neutrophils as the major inflammatory cell in a 38% of a strictly selected asthmatic population.

Increase of neutrophils in bronchial lavage was associated to severe asthma[22, 23], to chronic asthma[8] and to exposure to low molecular weight agents in occupational asthma[24]. Although neutrophils have also been associated with less severe asthma[6-9], in most of these studies the selection of patients was not homogeneous and the critical inclusion criteria such as age, smoking, length of asthma, and the use of inhaled steroids that may *per se* increase the number of neutrophils, were not taken into consideration. In consequence, in the specialized bibliography[25] a standard criterion for classification of neutrophilic asthma has yet to be clearly established.

After the washout period, eosinophil numbers remained low in EP compared with the baseline value (p = 0.04), but neutrophils in NP group returned to baseline values when treated with montelukast (p = 0.4) (Fig. 2).

After six weeks of treatment a significantly increased level of CC16 was found only in EP (p = 0.03) considering the total treated samples in each subset (Fig. 3).

Regarding the effects of montelukast on neutrophils, we analyzed the level of LTE4 in supernatant of baseline induced sputum, and found that it was higher in NP than in the EP subset (p = 0.03) as shown in Fig. 4.

The population of stable neutrophilic predominant asthmatics identified here, exhibited no differences in FEV_1 values compared with the EP subset; however, the PEFV was higher in EP than in NP. In this respect, Virchow[26] showed that the level of eosinophil cationic protein in sputum correlates with parameters of airflow hyperactivity.

In our study we have corroborated the effects of budesonide and montelukast in depleting the number of eosinophils as described by others[9]. However, an important novel observation is that montelukast was able to deplete neutrophils while budesonide did not modify their numbers. It is difficult to explain this montelukast´s effect on neutrophils only through its antagonism of cysteinyl leukotriene receptor without direct effects on LTB_4 levels[10]. A recent publication[27] described montelukast as having a specific inhibiting effect on 5-lipoxigenase enzyme and it can thus inhibit the chemotaxis of neutrophils via LTB_4. Considering NP group response to cysteinil leucotrien, we evaluated LTE_4, the last metabolite of cysteinyl leukotriene, and found it to be significantly higher in this subset, probably associated to its physiopathogenic. The finding that the neutrophil population returned to baseline values after montelukast treatment washout suggests that this inflammatory phenotype is not a transient condition.

Although both treatments reduced the numbers of inflammatory cells, it is important to stress that only budesonide and not montelukast improved DSS and SR, a common finding in clinical practice. Perhaps it results from multiple mechanisms of action of budesonide.

The finding that CC16 baseline levels showed no differences between the groups, indicating a similar inflammatory state due in part to Clara cell metaplasia to mucous cells and the consequent depletion of CC16 content, as has been demonstrated in experimental asthma models[28, 29].

The increase of CC6 in EP group after anti-inflammatory treatment could be the result of mucous metaplasia reversion recovering their normal morpho-functional status as was demonstrated by electron microscopy[28].

The deficient response of NP patients to anti-inflammatory treatments suggests that the primary defect could lie in the decline Clara cell population or in their secretory capacity[30, 31]. The lack of secretor response to increase CC16 value is a disadvantage for NP subset.

A recent report[32] emphasized the importance of identifying factors which predict the development of severe asthma. In this respect, NP could correspond to an asthma phenotype with a poor prognosis. This hypothesis is supported by the work of Ohchi et al[33] who demonstrated that CC16 low expression influences negatively and predicts the risk of progression of the disease in sarcoidosis. These observations reinforced the protector and modulator role of the CC16 in the inflammatory mechanism of the respiratory tract.

One of the major weaknesses of this study was the lack of a control group; however the strict inclusions criteria and ethical considerations prevented us to include a control group. However, the differences found in the two different therapeutic arms let us affirm they were due to the specific effect of the anti-inflammatory treatments and not to a regression to the mean.

The results presented here demonstrate a clearly-defined neutrophil predominate subset in asthma, with distinct clinical-functional characteristics and specific response to treatment. Leukotriene pathway appeared to be involved in the pathofysiology. The anti-inflammatory CC16 protein in the respiratory tract may constitute a useful biomarker for asthma prognosis.

Conflict of interest statement: None to declare.

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[…]Dícese que un hombre de ahora, llamado Paracelso, ha cambiado y trastornado todo el orden de las reglas antiguas, sosteniendo que la medicina, hasta hoy, sólo ha servido para matar a los hombres. Creo que demostrará esto con facilidad, pero no creo que fuese gran discreción poner mi vida a prueba de su nueva experiencia. Como dice el precepto, no hemos de creer a todos, porque todos pueden hablar de cuantas cosas quieran. Una persona que profesa esas novedades y reformas físicas me decía no ha mucho que todos los antiguos habían errado notoriamente en la naturaleza y movimiento de los vientos, lo que haría tocar evidentemente y como con la mano si quería oírle. "Entonces –le respondí– los que navegaban según las leyes de Teofrasto, ¿iban hacia Occidente cuando se encaminaban hacia Levante? ¿Viajaban de lado o hacia atrás?" "Eso era cosa de suerte –replicó mi hombre–, lo esencial es que en todo caso se engañaban." Díjele que prefería en ese caso atenerme a los efectos antes que a la razón. Pero estas cosas chocan a menudo. […]

Michel de Montaigne (1533-1592)

Ensayos (Essais, 1580-1588-1595-póstumos) *Apología de Raimundo Sebond.* Libro II, p. 215-6. Traducción de Juan G de Luaces. Buenos Aires: Hyspamérica, 1984