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Title: Clinical, functional and inflammatory characteristics in patients with pauci-granulocytic stable asthma: comparison with different sputum phenotypes

Short title: Characteristics of pauci-granulocytic vs. other asthma phenotypes

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Abstract

Background: According to induced sputum cell count, four different asthma phenotypes have been recognised (eosinophilic, neutrophilic, mixed and pauci-granulocytic). The aim of the present study was to detect functional and inflammatory characteristics of patients with pauci-granulocytic asthma.

Methods: 240 asthmatic patients were categorised in the four phenotypes according to cell counts in induced sputum. All patients underwent pulmonary function tests, and measurement of FeNO. The levels of IL-8, IL-13, and ECP were also measured in sputum supernatant. Treatment, asthma control and the presence of Severe Refractory Asthma (SRA) were also recorded.

Results: Patients were categorized in the four phenotypes as follows: eosinophilic (40%), mixed (6.7%), neutrophilic (5.4%) and pauci-granulocytic (47.9%). Although ACT did not differ between groups ($p=0.288$) patients with pauci-granulocytic asthma had better lung function ($FEV_1\%$ pred) (median (IQR): 71.5(59.0-88.75) vs 69.0(59.0-77.6) vs 68.0(60.0-85.5) vs 80.5(69.7-95.0), $p=0.009$] for eosinophilic, mixed, neutrophilic and pauci-granulocytic asthma respectively, $p=0.009$). SRA occurred more frequently in the eosinophilic and mixed phenotype (41.6% and 43.7% respectively) and less frequently in the neutrophilic and pauci-granulocytic phenotype (25% and 21.7% respectively, $p=0.01$). FeNO, ECP and IL-8 were all low in the pauci-granulocytic, whereas as expected FeNO and ECP were higher in eosinophilic and mixed asthma, while IL-8 was higher in patients with neutrophilic and mixed asthma ($p<0.001$ for all comparisons). Interestingly, 14.8% of patients with pauci-granulocytic asthma had poor asthma control.

Conclusion: Pauci-granulocytic asthma most likely represents a “benign” asthma phenotype, related to a good response to treatment, rather than a “true” phenotype

of asthma. However, pauci-granulocytic patients that remain not-well-controlled despite optimal treatment represent an asthmatic population that requires further study for potential novel targeted interventions.

Key words

Asthma

Eosinophils

Induced Sputum

Neutrophils

Pulmonary Function

Introduction

Asthma has been defined as a chronic disorder of airway inflammation, characterized by variable and recurring symptoms, airflow obstruction, and bronchial hyperresponsiveness (1). It has been recognised that among asthmatic patients there is great heterogeneity regarding the disease onset, symptoms, pattern of inflammation and response to treatment, which has evolved the need of recognising different clinical phenotypes (2). When examining the pattern of airway inflammation using sputum analysis, asthmatic patients can be classified in four different inflammatory subtypes (3). Subjects with a sputum neutrophil proportion $\geq 61\%$ are classified as having neutrophilic asthma and those with an eosinophilic proportion $\geq 1-3\%$ are classified as having eosinophilic asthma (3-5). When both neutrophils and eosinophils are increased, patients can be classified as having mixed granulocytic asthma and finally, asthmatic patients with lower levels of both neutrophils and eosinophils are classified as having pauci-granulocytic asthma(3). The

characterization of patients according to the aforementioned phenotypes has been used with success for the optimization of the treatment of asthma (4, 6, 7)

In the recent years, focus has been given to the presence of either eosinophils and neutrophils in induced sputum. Thus, sputum eosinophilia, has been related to the presence of atopic symptoms and good response to inhaled corticosteroids (6, 8) while studies have shown that optimization of therapy based on the levels of sputum eosinophilia results in better control of asthma (4). On the other hand, the presence of neutrophils is usually related to exposure to air pollutants (such as cigarette smoke) or the presence of a viral or bacterial infection (9).

Although both eosinophilic and neutrophilic asthma have been extensively studied, data on pauci-granulocytic asthma, regarding expression of biomarkers, disease severity and response to therapy, is limited. The aim of the present study was to evaluate the characteristics of patients with pauci-granulocytic asthma compared to the eosinophilic, the neutrophilic and the mixed phenotypes.

Methods

Study design

240 patients with a diagnosis of asthma who were followed up in two asthma clinics of 2 tertiary hospitals (1st Respiratory Medicine Department, University of Athens, Sotiria Hospital, Athens, Greece, and 2nd Respiratory Medicine Department, University of Athens, “Attikon” Hospital, Chaidari, Greece) were included to the study. All patients were optimally treated for at least 6 months. After accepting to participate to the study, their demographic and functional characteristics including smoking status, atopy, body mass index (BMI) and current medication were recorded. The level of the dose of ICS (low, medium and high) was also recorded

based on the GINA Guidelines 2016 (1). All patients underwent spirometry tests, measurement of Fraction of exhaled Nitric Oxide (FeNO), and sputum induction in order to assess sputum cell counts and inflammatory biomarkers in induced sputum. Asthma control was evaluated using the Asthma Control Test (ACT). All tests were performed in the morning and all patients had received their medication before. The study protocol was approved by the ethics committees of both hospitals and all subjects provided written informed consent.

Measurement of Pulmonary Function, Atopy and exhaled Nitric Oxide

Post Bronchodilation spirometry was performed with the use of Master Screen Body (Viasys Healthcare, Jaeger, Hoechberg, Germany) according to the American Thoracic Society guidelines (10) and was evaluated by asthma specialists. Atopy was defined as the presence a positive skin prick test to at least one out of 20 common aeroallergens. FeNO was measured using a portable NO analyser (NIOX MINO, Aerocrine, Solna, Sweden) (11). MINO measurements are performed at a fixed flow rate of 50 mL/sec, are expressed as parts per billion (ppb) and are in clinically acceptable agreement with measurements provided by a stationary analyzer according to the ATS guidelines (11-13).

Sputum induction and processing

Sputum induction was performed as previously described (14, 15), using all the modifications for safe measurements according to the underlying asthma severity (16, 17). Sputum supernatant was removed with centrifugation and total cell count was evaluated with a haemocytometer using Trypan Blue stain. Slides were prepared by cytopsin (Shandon, Runcorn, UK) and were stained with May-Grunwald

and Giemsa for differential cell counts. A blind observer performed counting of a minimum 500 inflammatory cells in each sample. Sputum samples were considered acceptable if they had a volume of at least 2 ml after final expectoration and the percentage of squamous cells on the prepared slides was <10%. Total cell count was expressed as the number of cells $\times 10^6/\text{ml}$ and sputum inflammatory cells as percentage (%) of the non-squamous cells. Sputum supernatants were stored at -70°C until measurement.

Definition of asthma phenotypes according to the sputum cell count

Patients were classified in four different groups (phenotypes) according to the percentage of eosinophils and neutrophils in induced sputum. The neutrophilic phenotype was defined by the presence of $\geq 60\%$ neutrophils and $< 3\%$ eosinophils, the eosinophilic phenotype by $\geq 3\%$ eosinophils and $< 60\%$ neutrophils, the mixed phenotype by the presence of $\geq 3\%$ eosinophils and $\geq 60\%$ neutrophils and the paucigranulocytic by both $< 3\%$ eosinophils and $< 60\%$ neutrophils (3).

Measurement of biomarkers in induced sputum

Levels of interleukin (IL)-13, IL-8, and eosinophilic cationic protein (ECP) in sputum supernatant were measured using commercially available kits. ECP was measured using Unicap ECP kit (lower limit of detection: 0.5ng/ml, Pharmacia diagnostics Upsala Sweden), while IL-13 and IL-8 were measured using enzyme-linked immunosorbent assay kits (ELISA, R&D systems, Minneapolis, Minnesota, USA). The lower limits of detection were 32 pg/ml and 3.5 pg/ml, for IL-13 and IL-8 respectively.

Assessment of asthma control and Severe Refractory Asthma

Asthma control was assessed according to GINA guidelines(1) by two asthma experts (SL and PB). A validated questionnaire, the Asthma Control Test (18) was additionally used. Presence of Severe Refractory Asthma (SRA) was assessed according to the ATS workshop consensus (19).

Statistical analysis

Categorical variables are presented as n (%), whereas numerical variables are presented as mean $\pm$ standard deviation (SD) or median (interquartile ranges) for normally distributed and skewed data, respectively. Normality of distributions was checked using Kolmogorov-Smirnov test. Comparisons between different asthma phenotypes according to the cell count in induced sputum were performed using chi-square tests for categorical data, as well as One-way ANOVA or Kruskal-Wallis test for normally distributed or skewed numerical data, respectively. All p-values <0.05 were considered statistically significant. Data were analysed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Demographic and functional characteristics of the study participants are shown on Table 1. The four different phenotypes did not differ regarding their age, gender, atopy, and smoking status. Patients were categorized in the four different phenotypes as follows: eosinophilic phenotype 96/240 (40%), mixed phenotype 16/240 (6.7%), neutrophilic phenotype 13/240 (5.4%) and paucigranulocytic phenotype 115/240 (47.9%) (Figure 1).

Lung function, asthma severity and treatment requirements

Although, ACT scores did not differ among four groups ($p=0.288$), pauci-granulocytic asthma had better lung function based on post bronchodilation FEV₁ (% pred.) [median (IQR): 71.5 (59.0-88.75) vs 69.0 (59.0-77.6) vs 68.0 (60.0-85.5) vs 80.5 (69.7-95.0), $p=0.009$], and FEV₁/FVC ratio 67.5 (60.0, 77.0) vs 66.0 (63.0, 74.3) vs 65.0 (60.0, 72.0) vs 71.0 (66.8, 76.0), $p=0.041$, for eosinophilic, mixed, neutrophilic and pauci-granulocytic asthma respectively (Figure 2 A and B). Furthermore, patients with pauci-granulocytic asthma required less often high doses of ICS (44.8% vs 37.5% vs 23.0% vs 21.7%, for eosinophilic, mixed, neutrophilic and pauci-granulocytic phenotype, respectively), $p=0.004$. There were no differences in the use of LABA, oral corticosteroids or LTRA between the groups. Finally, SRA occurred more frequently in the eosinophilic and mixed phenotype (41.6% and 43.7% respectively) and less frequently in the neutrophilic and pauci-granulocytic phenotype (25% and 21.7% respectively), $p=0.01$.

Inflammatory biomarkers in the four asthma phenotypes

As expected, FeNO and ECP were higher in eosinophilic and mixed asthma while IL-8 was higher in patients with neutrophilic and mixed asthma ($p<0.001$ for all comparisons) Figure 3 (A-C). In our study, the levels of IL-13 did not differ among the four groups ($p=0.585$). The levels of each biomarker in the four different asthma phenotypes of our study are shown in Table 2. Furthermore, Table 2 shows the different levels of each biomarker according to the presence of characteristics of Severe Refractory asthma (SRA). Patients with SRA presented higher levels of FeNO compared to those with mild/moderate asthma in the eosinophilic and pauci-

granulocytic phenotypes; higher levels of sputum IL-13 in the eosinophilic, mixed and pauci-granulocytic phenotypes; and higher levels of sputum IL-8 in patients with pauci-granulocytic asthma.

Discussion:

In this study we have shown that pauci-granulocytic asthma represents the majority of well treated asthmatic patients in our tertiary centres. These patients are characterized by better lung function, while they are less often characterised as suffering from SRA and need for high doses of ICS. Finally, patients with pauci-granulocytic asthma express lower levels of inflammatory biomarkers in exhaled air and induced sputum supernatants, representing a less intense inflammatory process.

The identification of the type of airway inflammation can be very helpful for making the optimal treatment decision. Sputum differentials can be clinically relevant, especially in the context of the identification of sputum eosinophilia in appropriate patients with severe asthma in centers experienced in this technique and this has been recently proposed by the ERS/ATS guidelines on severe asthma. The presence of eosinophils in induced sputum is an important prognostic factor of response to therapy with inhaled corticosteroids (6, 9). In this spectrum, the absence of eosinophils (i.e. in either the neutrophilic or the pauci-granulocytic phenotype) in asthmatic patients is probably related to treatment with adequate dose of inhaled corticosteroids. Interestingly, in our study, patients with pauci-granulocytic asthma had better lung function and received less frequently high doses of ICS which leads to the conclusion that in these patients, asthma was either adequately controlled with lower doses of inhaled corticosteroids, or that the physician was able to perform a

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step down in asthmatic therapy due to total control of asthma according to the GINA guidelines(1). This is further supported by the fact that pauci-granulocytic patients with SRA presented higher levels of FeNO, and sputum IL-13 and IL-8 compared to those with mild/moderate disease. The presence of surrogate inflammatory markers in the absence of cellular inflammation supports the need for a more comprehensive approach in the evaluation of airways inflammation in patients with SRA. There is limited knowledge on the clinical relevance and the potential therapeutic implications of the identification of patients with non-eosinophilic inflammation and our study provides some additional evidence in that direction.

Persistence of sputum eosinophilia despite therapy (i.e. in the cases of the eosinophilic and mixed phenotypes) might represent reduced compliance, mishandling of the inhaler device, continuous exposure to allergens or inadequate dose of inhaled corticosteroids. However, in some patients the presence of persistent eosinophilic inflammation may represent a reduced response to ICS (9).

Although in our study pauci-granulocytic asthma was related to better lung function and lower need of high doses of ICS, still 25 out of 115 patients (i.e. 21.7%) were characterized as having SRA while in 17 out of 115 patients (i.e. 14.8%) had an ACT score of ≤ 19 leading to the conclusion that their asthma was not well controlled despite the absence of inflammatory cells in their induced sputum. This observation is in accordance to the hypothesis that in some cases, pauci-granulocytic asthma may involve intrinsic alteration in structural cell function (such as epithelial, smooth muscle cells, vessels and nerves) which might orchestrate airway inflammation (20, 21). These patients may present steroid insensitivity and therefore the use of high-dose ICS will not provide any additional benefit in their management. This sub-population of non-controlled patients with pauci-granulocytic asthma requires further

study and the identification of potential novel targeted treatments focusing on structural cells.

However, in some patients with pauci-granulocytic asthma, the absence of inflammatory cells, could possibly be the result of a pre-existing eosinophilic asthma adequately treated with ICS in which there is no neutrophilic inflammation. This hypothesis might also explain the better lung function and absence of criteria of SRA observed in the majority of this group of patients. In other words, in certain patients the presence of pauci-granulocytic asthma, might represent a treatment success and could be considered as a result of the successful therapeutic intervention rather than a different asthma phenotype.

On the other hand, neutrophilic inflammation is usually related to environmental factors such as air pollutants (22) or infections and bacterial colonization (23). Patients with neutrophilic asthma present steroid insensitivity while neutrophilic inflammation is often related to progressive loss of lung function and persistent airflow limitation (24). Interestingly, in our study, patients with neutrophilic inflammation (either in the neutrophilic or in the mixed phenotype), had worse lung function and more frequently suffered from SRA and received high doses of ICS, the latter probably prescribed in an effort to control the patient's persistent symptoms. It is also important to mention that in our study only a minority of patients had neutrophilic inflammation (i.e. were categorized in the neutrophilic and the mixed phenotype) and a possible explanation for this finding should be investigated. Moreover, the limited response of patients with sputum neutrophilia to ICS (25) provides an additional implication for the appropriate identification of the sputum patient endotype in experienced centers. The limited evidence for potential benefit from macrolide antibiotics in patients with neutrophilic (26) or non-eosinophilic (27)

asthma, was not confirmed in a more recent study (28) highlighting an unmet clinical need for this population if appropriately characterized by induced sputum.

Our study has several limitations. First, all our patients were optimally treated and stable for at least 6 months. Thus, we do not have information regarding their airway inflammation at the time of disease onset, before treatment initiation. This fact does not allow us to have a specific picture on what kind of inflammation patients had before taking ICS to allow a safe conclusion on whether the neutrophilic and the pauci-granulocytic phenotype represents eosinophilic asthma that has responded to treatment. Furthermore, for the same reason we do not have information about the type of inflammation during symptom deterioration or during the development of an asthma exacerbation. This limitation is present in all four group of our patients including patients with pauci-granulocytic asthma in which we also do not have data on whether their inflammatory profile would alter in the case of symptom deterioration and loss of asthma control since it has been reported that sputum inflammatory profile in patients with moderate and severe asthma usually changes and remains stable only in approximately one third of patients (29).

In conclusion, in the majority of well-treated asthmatics pauci-granulocytic asthma represents a “benign” asthma phenotype that is related to a relatively good response to treatment, rather than a “true” phenotype of asthma. The pauci-granulocytic patients that remain not-well-controlled despite optimal treatment represent an asthmatic population that requires further study for potential novel targeted interventions.

Conflict of interest: Konstantinos Kostikas is a Novartis Employee. All other authors declare that they do not have conflict of interest related to the present manuscript.

Authors Contributions:

PN, AP, KK and SL wrote the manuscript. EP, GH and PN have performed sputum induction and cell count. SL, PB, SP and NK have designed the study and provided the patients who have participated. GP have performed all measurements of the biomarkers and contributed to the writing of the methods section and analysis of data. AP, PN KK and SL have performed the statistical analysis. All authors have read and approved the final version of the manuscript.

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Table 1: Demographic and functional characteristics of the study groups

Parameters	Eosinophilic (n=96)	Mixed (n=16)	Neutrophilic (n=13)	Pauci- granulocytic (n=115)	p value
Age (years)	51.5±14.3	57.5±10.2	56±12.5	54±13.03	0.374
Gender (Female)	68 (70.8)	10 (62.5)	9 (69.2)	82 (71.3)	0.910
Atopy N(%)	51 (53.1)	7 (43.8)	4 (30.8)	48 (41.7)	0.256
BMI (kg/m ²)	26.7 (24.4, 30.0)	28.9 (27.0, 32.8)	27.5 (24.2, 32.3)	27.0 (25.0, 30.0)	0.098
Smoking (current smokers) N(%)	18 (18.8)	5 (31.3)	2 (15.4)	18 (15.7)	0.544
FEV ₁ % pred.	71.5 (59.0-88.7)	69.0 (59.0-77.6)	68.0(60.0-85.5)	80.5 (69.7-95.0)	0.009
FEV ₁ /FVC (%)	67.5 (60.0, 77.0)	66.0 (63.0, 74.3)	65.0 (60.0, 72.0)	71.0 (66.8, 76.0)	0.041
ACT score	22 (19-24)	23 (20-24)	23 (19-24)	23 (21-24)	0.288
Not well-controlled asthma (ACT ≤19) N(%)	24 (25.0)	3 (18.8)	3 (23.1)	17 (14.8)	0.311
SRA N(%)	40 (41.7)	7 (43.8)	3 (23.1)	25 (21.7)	0.011
High dose of ICS N(%)	43 (44.8)	6 (37.5)	3 (23.1)	25 (21.7)	0.004
LABA N(%)	86 (89.6)	14 (87.5)	11 (84.6)	97 (84.3)	0.050
Oral CS N(%)	25 (26.0)	5 (31.3)	3 (23.1)	19 (16.5)	0.289
LTRA N(%)	22 (22.9)	3 (18.8)	1 (7.7)	12 (10.4)	0.076

Abbreviations: FeNO: Fraction of exhaled Nitric Oxide, ppb: parts per billion, FEV₁: Forced Expiratory Volume in one second, FVC: Forced exhaled Vital Capacity, ACT: Asthma Control Test, SRA: Severe Refractory Asthma, ICS: Inhaled Corticosteroids, LABA: Long Acting Beta Agonists, CS: corticosteroids, LTRA: Leukotriene Receptor Antagonists
Data are presented as Mean (SD) or Median (IQR) unless other indicated.
Bold indicated statistically significant differences among study groups

Table 2

Levels of Biomarkers in induced sputum in the four asthma phenotypes

Biomarker	Eosinophilic	Mixed	Neutrophilic	Pauci-granulocytic	p-value
FeNO (ppb)					
All	27.0 (19.3, 57.0)	21.5 (12.0, 35.0)	17.0 (12.5, 25.5)	19.0 (12.7, 24.0)	<0.001
SRA	43.0 (23.5, 67.8)*	22.0 (12.0, 26.0)	22.0 (20.0, 22.0)	22.0 (21.0, 26.0)*	0.002
Mild/moderate asthma	24.0 (17.0, 38.8)*	21.0 (11.0, 41.0)	14.0 (12.0, 23.5)	17.0 (12.0, 23.0)*	0.002
IL-13 (pg/ml)					
All	87.0 (66.0, 187.0)	84.5 (59.5, 205.7)	78.0 (59.5, 95.0)	89.0 (71.0, 109.0)	0.585
SRA	179.0 (68.8, 212.8)*	205.0 (82.0, 312.0)*	92.0 (89.0, 92.0)	109.0 (90.0, 199.0)*	0.867
Mild/moderate asthma	76.0 (65.0, 102.0)*	70.0 (44.0, 87.0)*	76.0 (59.5, 88.0)	87.0 (68.0, 103.3)*	0.116
ECP (ng/ml)					
All	1.8 (0.9, 2.6)	1.8 (1.5, 2.6)	0.9 (0.7, 1.2)	0.9 (0.7, 1.1)	<0.001
SRA	1.9 (0.9, 3.4)	2.6 (1.5, 3.1)	1.4 (0.9, 1.4)	0.9 (0.7, 1.2)	<0.001
Mild/moderate asthma	1.7 (1.1, 2.1)	1.5 (1.1, 2.1)	0.8 (0.6, 1.1)	0.7 (0.9, 1.1)	<0.001
IL-8 (ng/ml)					
All	0.9 (0.6, 2.1)	3.8 (1.6, 3.9)	4.8 (3.1, 5.1)	0.8 (0.6, 1.7)	<0.001
SRA	1.3 (0.7, 2.7)	3.7 (1.6, 3.9)	4.8 (4.1, 4.8)	1.1 (0.7, 2.5)*	0.003
Mild/moderate asthma	0.8 (0.6, 2.1)	3.9 (1.3, 4.0)	5.0 (2.7, 5.3)	0.8 (0.5, 1.5)*	<0.001

Abbreviations: FeNO: Fraction of Exhaled Nitric Oxide, ppb: parts per billion, IL-13: Interleukin 13, ECP: Eosinophilic Cationic Protein, IL-8: Interleukin 8.

Data are presented as Median (IQR)

Bold indicated statistically significant differences among study groups

* indicates differences between patients with and without SRA for the same study group.

Figure Legends

Figure 1:

Representation of asthma phenotypes according to induced sputum cell count in our study participants

Figure 2

Post bronchodilation **A.** FEV₁ and **B.** FEV₁/FVC in patients with different asthma phenotypes based on induced sputum cell counts

Figure 3

Differences in **A.** FeNO, **B.** ECP in sputum supernatant, and **C.** IL-8 in sputum supernatant among study groups







