



# Serum levels of IL-5, IL-6, IL-8, IL-13 and IL-17A in pre-defined groups of adult patients with moderate and severe bronchial asthma

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## ARTICLE INFO

### Keywords:

Moderate and severe asthma

IL-5

IL-6

IL-8

IL-13

IL-17A

## ABSTRACT

**Background:** Bronchial asthma (BA) is a complex disease characterised by persistent inflammation. Exhaled nitric oxide (FeNO) and blood eosinophil count (b-Eos) are biomarkers for type 2 endotype of BA.

**Objective:** To analyse a panel of serum interleukins and total IgE in predefined by FeNO and b-Eos groups of moderate and severe BA patients.

**Methods:** Serum levels of IL-5, IL-6, IL-8, IL-13 and IL-17A (ELISA) were measured in 30 healthy controls (HC) and 80 adult BA patients. BA patients were split into 4 groups. Group 1:Low FeNO/Low b-Eos (n = 23; 28.8%); Group 2:Low FeNO/High b-Eos (n = 17; 21.3%); Group 3:High FeNO/Low b-Eos (n = 15; 18.8%); Group 4:High FeNO/High b-Eos (n = 25; 31.3%).

**Results:** All interleukins and total IgE were significantly higher in patients with BA as compared with HC. IL-5 levels were highest in Group 2 (p < 0.05). IL-6, IL-13 and IL-17A levels were elevated in Groups 2, 3 and 4 as compared with HC (p < 0.05). Higher IL-8 levels were associated with a pattern of current smokers. Highest IL-17A levels were found in type 2 high groups with frequent exacerbations, mostly uncontrolled and severe BA. We have found a distinct pattern for each group based on demographic, clinical, functional, immunological and inflammatory characteristics.

**Conclusion:** FeNO and b-Eos are useful in the identification of severe type 2 BA subgroups with frequent exacerbations. IL-5, IL-6, IL-13 and IL-17A are involved in the persistent type 2 immune response in moderate and severe BA. We have identified a pattern of refractory, severe type 2/IL-17A high BA in the real clinical practice.

## 1. Introduction

Bronchial asthma (BA) is a chronic disease characterized by pathophysiological heterogeneity [1]. The cells interactions are driven by the intercellular signalling system of cytokines and chemokines. Different approaches for identification of phenotypes were developed, to better understand the pathogenesis of BA. Despite the successful identification of distinct phenotypic features, there is a significant overlap in the inflammatory characteristics among the different groups of Pts. The variety of phenotypes is greater and better studied in Pts with severe BA [2–4]. Two major endotypes of BA were described in the last years – type 2 and non-type 2 BA. In 50–70% of the cases are found characteristics for type 2 immune response [5,6]. Most commonly used

biomarkers for type 2 BA are blood eosinophils (b-Eos) and fraction of exhaled nitric oxide (FeNO) [7].

The use of serum interleukins (ILs) for assessment of airway inflammation is limited, because they are predominantly local mediators. For this reason, the results obtained from an induced sputum and bronchoalveolar lavage (BAL) fluid (invasive and time consuming procedures) may be accepted as more reliable. On other hand we suggest that there may exist a systemic inflammatory reaction, detectable in serum samples in therapy resistant BA, that may cause persistence of the local inflammation despite the maintenance inhalatory therapy. In this scenario the measurement of serum interleukins in combination with other biomarkers may be useful strategy in assessment of severe, therapy resistant BA subgroups.

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<https://doi.org/10.1016/j.rmed.2019.06.024>

Received 18 February 2019; Received in revised form 5 June 2019; Accepted 25 June 2019

Available online 26 June 2019

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## Abbreviations

|       |                                       |
|-------|---------------------------------------|
| ACT   | Asthma Control Test                   |
| ACQ   | Asthma Control Questionnaire          |
| AE    | Acute Exacerbation                    |
| ATS   | American Thoracic Society             |
| BA    | Bronchial Asthma                      |
| BAL   | Bronchoalveolar Lavage                |
| BMI   | Body Mass Index                       |
| b-Eos | Absolute Blood Eosinophilic Count     |
| b-Neu | Absolute Blood Neutrophilic Count     |
| CD    | Cluster of Differentiation            |
| COPD  | Chronic Obstructive Pulmonary Disease |
| ELISA | Enzyme-linked Immunosorbent Assay     |
| ERS   | European Respiratory Society          |
| FeNO  | Fraction of Exhaled Nitric Oxide      |

|      |                                       |
|------|---------------------------------------|
| FEV1 | First Second Forced Expiratory Volume |
| FVC  | Forced Vital Capacity                 |
| GINA | The Global Initiative for Asthma      |
| GLI  | Global Lung Function Initiative       |
| HC   | Healthy Controls                      |
| Ig   | Immunoglobulin                        |
| ICS  | Inhaled Corticosteroids               |
| ILs  | Interleukins                          |
| ILC  | Innate Lymphoid Cells                 |
| LLN  | Lower Limit of Normal                 |
| OCS  | Oral Corticosteroids                  |
| Pts  | Patients                              |
| Ppb  | Parts per Billion                     |
| SPT  | Skin Prick Test                       |
| Th   | T-helper                              |

Different studies are showing detectable levels of ILs in BAL [8], sputum [9] and also in serum [10–12]. We did not manage to found study with direct comparisons of ILs levels in BAL/sputum and serum from same population of BA Pts, but two studies of Akiki et al. [11] and Liang et al. [12] are confirming the existence of separate serum ILs patterns in Pts with BA. They also suggest that the measurement of ILs in blood may be useful in assessment of severe BA.

We have used a panel of cytokines for this study that include IL-5, IL-6, IL-8, IL-13 and IL-17A. IL-5 and IL-13 are two of the mostly discussed type 2 cytokines, produced by T-helper type 2 (Th2) cells and innate lymphoid cells 2 (ILC2) [14,15]. IL-5 plays a central role in stimulating the growth, differentiation, and maturation of eosinophils in the bone marrow, as well as in their accumulation, activation, and survival in tissues [16]. IL-13 is involved in class-switch recombination of B-cells to IgE production [17,18]. IL-6 is one of the best researched proinflammatory cytokines in many chronic diseases and the studies have shown that it is also elevated in serum and sputum of Pts with BA [19–21]. IL-6 levels may be affected by viral infections and obesity, two important factors in exacerbations and severity of BA [21]. There are data about involvement of IL-17A in the pathogenesis of asthma [22] and a possible positive feedback between IL-6 and IL-17A is also suggested [23]. IL-17A orchestrate the neutrophilic influx into the airways [22], but on other hand there are growing evidence that IL-17A enhance Th2 mediated immune response [10,24,25]. Other potent chemoattractant of neutrophils in the airways is IL-8 [26], found to be elevated in serum [27], BAL [8] and sputum of treatment-refractory severe asthma [28].

We think that the classification of Pts with moderate and severe BA from the real clinical practice, according to the presence or absence of systemic and local type 2 inflammatory response may give us information about the complex immunological profile of each group.

The aim of this study was to analyse a panel of serum interleukins and total IgE in pre-defined by FeNO and b-Eos subgroups of moderate and severe BA patients.

## 2. Methods

### 2.1. Subjects

This study was done with permission of the Ethics Committee on Medical Research of the Medical University – Sofia. All Pts were at age over 18 years and participate voluntarily after informed consent.

In the study were included 80 adult Pts with moderate and severe BA out of exacerbation and on maintenance asthma therapy (Step 3, 4 and 5 according to GINA) in the last four weeks before participation in the study and 30 healthy non-atopic controls (HC) matched for gender, age, body mass index (BMI) and smoking status. The tests were

conducted in the ordinary course of ambulatory practice for a period of 2 years. The diagnosis and severity of BA were determined according to the international guidelines – GINA, ATS/ERS 2014 [29,30]. The exclusion criteria for participation were exacerbation in the last 4 weeks prior to the study, active infection, interstitial lung disease, malignancy, heart, autoimmune, endocrinological or bone diseases, certain immune deficiency, pregnancy, biological treatment. Current and ex-smokers with a history of asthma were also included if the patient had at least 12% and 200 ml improvement in FEV<sub>1</sub> after inhalation of 400 µg of Salbutamol at the time of inclusion.

#### 2.1.1. Methods

All participants have completed questionnaires that assessed demographic data, medical history, comorbidities, medication use, number of exacerbations during the last year, imposed intake of systemic corticosteroids, and/or hospitalization or emergency room visit due to BA. Symptoms and asthma control were measured, with validated asthma questionnaires (GINA four questions, Asthma control test (ACT), Juniper Asthma Control Questionnaire (ACQ 5, 6 and 7). Asthma acute exacerbation (AE) was defined by hospital or emergency admission due to respiratory problems or use of oral corticosteroids (OCS) for breathing difficulties in the last year. Detailed physical examination was done. Measurement of the fraction of exhaled nitric oxide (FeNO) was done by a system NIOX MINO (Aerocrine, Sweden) [31]. Pre- and postbronchodilator (400 µg of Salbutamol) lung functional parameters were assessed with standard forced spirometry (MiniSpir, software WinspiroPRO version 6.8.0; Italy) based on the recommendation of Global Lung Function Initiative (GLI). Venous blood samples were collected from each subject (with BA and HC) at the same time of the day after fasting. Serum was separated after clotting and centrifugation for 10 min at 1000 x g and the samples were stored at a temperature of –80 °C. Total serum IgE levels (EUROIMMUN total IgE ELISA, Germany) were measured and levels of 100 IU/ml were accepted as upper limit of reference range for adults. Absolute eosinophil count in peripheral blood was evaluated by manual differentiation of the leucocytes. Skin prick tests (SPT) were performed with the most common aeroallergens - house dust mites, pollen, grasses, molds, weeds, cat, dog, guinea pig, feathers. Serum levels of IL-5, IL-6, IL-8, IL-13, IL-17A were measured by enzyme-linked immunosorbent assay (ELISA) (Diacclone, France) according to the manufacturer's instructions. IL-8 was measured in 70 of the BA Pts.

#### 2.1.2. Data statistical analysis

All statistical analyses were performed using SPSS Software, version 19.0 (IBM, SPSS statistics Inc.). The categorical variables were presented in percentages and quantitative as mean and standard deviation (SD) or median and interquartile ranges (IQR). Analysis of variance

(ANOVA) and independent-samples T-test were used for normally distributed data, and Kruskal-Wallis and Mann-Whitney U tests were used for data with different than normal distribution. Person's chi-square ( $\chi^2$ ) test was used to test for differences in categorical variables. Correlations between data were analysed by Spearman correlation test. P-value under 0.05 was considered as statistically significant. We have used standardization of the measurements by z score in Fig. 3 for more clear visualization.

### 2.1.3. BA and HC main characteristics

The main characteristics of BA and HC groups are shown on Table 1. The HC were non-atopic, with normal lung function parameters and no history or symptoms of lung diseases or any other severe co-morbidity. They were matched by gender, age, BMI and pack-years.

### 2.1.4. Group selection

BA Pts were selected according to FeNO levels ( $< 30$  and  $\geq 30$  ppb) and b-Eos ( $< 300$  and  $\geq 300$  cells/microL) into four groups: Group 1 - Low FeNO/Low b-Eos ( $n = 23$ ; 28.8%); Group 2 - Low FeNO/High b-Eos ( $n = 17$ ; 21.3%); Group 3 - High FeNO/Low b-Eos ( $n = 15$ ; 18.8%); Group 4 - High FeNO/High b-Eos ( $n = 25$ ; 31.3%)

## 3. Results

Thirty-five (43.8%) of the BA Pts have met the criteria for severe BA (ATS/ERS 2014). Fifty-six (70%) of the BA Pts had an AE in the

previous year and 22 (27.5%) had 2 or more AEs. According to GINA criteria for control of symptoms, we have found complete control in 36 (45%) Pts, partial control in 20 (25%) and poor control in 24 (30%) of the Pts. When we have use ACT, the results were as follows: well controlled – 43 (53.8%), partly controlled – 22 (27.5%) and poor controlled – 15 (18.8%). According to ACQ7, well controlled ( $< 0.75$  scores) were 32 Pts (40%), Pts in “gray zone” (0.75–1.50 scores) were 16 (20%) and uncontrolled ( $> 1.50$  scores) - 32 (40%) Pts.

The median level of total serum IgE was 119.95 (42.30–289.51) IU/ml with a minimum value of 0.1 IU/ml and maximum - 1983 IU/ml. The median level of FeNO was 29.5 (18.3–47.8) ppb. The minimum measured value was 7 ppb and the maximum - 178 ppb. The median of b-Eos was 322 (205–509) cells/microL with minimum levels of 41 cells/microL and maximum - 2125 cells/microL.

We have found significantly higher FeNO in non-smokers (31 (21–60) ppb) and ex-smokers (31 (19–60) ppb), compared to current smokers (20 (10–29) ppb) ( $\chi^2 = 7.286$ ,  $p = 0.026$ ). FeNO was significantly elevated in more severe BA Pts on moderate/high dose ICS and low dose OCS (step 4 and 5) (30 (21–45) ppb and 51 (31–75) ppb, as follows) compared with those on low dose ICS (step 3) (21 (16–43) ppb) ( $\chi^2 = 10.097$ ,  $p = 0.006$ ). We have observed elevated FeNO in Pts with more than 1 exacerbation in the last year (44 (30–68) ppb), preFEV1/FEV  $< LLN$  (35 (22–68) ppb) and b-Eos  $\geq 300$  cells/microL (37 (22–67) ppb) ( $p < 0.05$  for all comparisons).

We have found significantly higher b-Eos in uncontrolled (413 (272–517) cells/microL) and severe BA (470 (290–651) cells/microL)

**Table 1**  
Main characteristics of all participants.

|                                  | BA N = 80                     | HC N = 30               | p-value   |
|----------------------------------|-------------------------------|-------------------------|-----------|
| Age, years (Range)               | 45.2 $\pm$ 14.2 (19–74)       | 43.4 $\pm$ 13.3 (18–66) | 0.516     |
| Female/male                      | 52/28 (65/35%)                | 22/8 (73.3/26.7%)       | 0.407     |
| BMI, kg/m <sup>2</sup>           | 26.7 (22.8–30.7)              | 25.3 (21.6–28)          | 0.280     |
| Obesity, no/yes                  | 57/23 (71.3/28.8%)            | 6 (20%)                 | 0.602     |
| Smoking history, no/ex/current   | 38/23/19 (47.50/28.75/23.75%) | 17/6/7 (56.7/23.3/20%)  | 0.606     |
| Pack-years                       | 3.6 (0–11)                    | 0 (0–7)                 | 0.349     |
| Onset, age                       | 33.3 $\pm$ 12                 | -                       | -         |
| Onset, groups $< 20/20-40/ > 40$ | 22/29/29 (27.5/36.25/36.25%)  | -                       | -         |
| Duration, years                  | 8.50 (3–18.75)                | -                       | -         |
| SPTs, neg/mono/poly              | 19/19/42 (23.8/23.8/52.5%)    | -                       | -         |
| Rhinitis, no/yes                 | 19/61 (23.7/76.3%)            | -                       | -         |
| ACQ5 score                       | 1.00 (0.20–2.15)              | -                       | -         |
| ACQ7 score                       | 1.00 (0.32–2.25)              | -                       | -         |
| ACT score                        | 21 (16–24)                    | -                       | -         |
| Uncontrolled (GINA)              | 44 (55%)                      | -                       | -         |
| AE/last 1 y                      | 1 (0–2)                       | -                       | -         |
| AE $> 1$ /last y                 | 56 (70%)                      | -                       | -         |
| Severe BA (ATS/ERS)              | 35 (43.8%)                    | -                       | -         |
| Therapy step 3/4/5               | 22/48/10<br>(27.5/60/12.5%)   | -                       | -         |
| Use of low dose OCS              | 10 (12.5%)                    | -                       | -         |
| Total IgE, IU/ml                 | 119.95 (42.30–289.51)         | 5.85 (2.10–15.50)       | $< 0.001$ |
| Total IgE $> 100$ IU/ml          | 44 (55%)                      | 0                       | -         |
| FeNO, ppb                        | 29.5 (18.3–47.8)              | 10 (6.8–13.3)           | $< 0.001$ |
| b-Eos, cells/microL              | 322 (205–509)                 | 105 (79–185)            | $< 0.001$ |
| b-Neu, 10 <sup>9</sup> /L        | 4.65 (3.51–5.94)              | 4.45 (3.25–4.90)        | 0.101     |
| preFVC, Z score                  | −0.94 (−1.88; −0.07)          | 0.73 (0; 1.13)          | $< 0.001$ |
| preFEV1, Z score                 | −1.49 (−2.78; −0.49)          | 0.40 (−0.53; 1.23)      | $< 0.001$ |
| preFEV1/FVC, Z score             | −1.35 (−2.30; 0.04)           | −0.06 (−0.50; 0.37)     | $< 0.001$ |
| postFVC, Z score                 | −0.61 (−1.42; 0.05)           | -                       | -         |
| postFEV1, Z score                | −1.13 (−2.20; −0.16)          | -                       | -         |
| postFEV1/FVC, Z score            | −0.94 (−1.86; 0.27)           | -                       | -         |
| postFEV1/FVC $< LLN$             | 25 (31.3%)                    | -                       | -         |
| Change in FEV1, %                | 5 (0–13.50)                   | -                       | -         |

Results are presented as number (%), mean  $\pm$  standard deviation (*Italic*), median and interquartile range (IQR); The p-value is obtained by Student's t-test or Mann-Whitney U test. Categorical variables are analysed by chi-square test.

Abbreviations: AE – acute exacerbations; b-Eos – blood eosinophils; b-Neu – blood neutrophils; OCS – oral corticosteroids; SPTs – skin prick tests; ACT – asthma control test; ACQ – asthma control questionnaire; FeNO – fraction of exhaled nitric oxide; FVC – forced vital capacity; FEV1 – first second forced expiratory volume; GINA – The Global Initiative for Asthma; BMI – body mass index; ATS – American Thoracic Society; ERS – European Respiratory Society; ppb – parts per billion; LLN – Lower Limit of Normal.

( $p < 0.05$  for all comparisons). B-Eos were also higher in Pts with  $\text{FeNO} > 50$  ppb (462 (385–792) cells/microL). B-Eos were significantly elevated in more severe BA Pts on high dose ICS and low dose OCS (step 5) (645 (294–1106) cells/microL), compared with Pts on therapy step 3 and 4 (245 (124–400) cells/microL and 345 (220–511) cells/microL as follows) ( $\chi^2 = 8.662$ ,  $p = 0.013$ ).

We have found the following correlations (Fig. 1):

- $\text{FeNO}$  and b-Eos ( $r = 0.298$  (95%CI 0.084; 0.486),  $p = 0.007$ )
- $\text{FeNO}$  and preFEV1 ( $r = -0.233$  (95%CI -0.431; -0.013),  $p = 0.038$ )
- $\text{FeNO}$  and preFEV1/FVC ( $r = -0.284$  (95%CI -0.474; -0.068),  $p = 0.011$ )
- $\text{FeNO}$  and postFEV1/FVC ( $r = -0.260$  (95%CI -0.454; -0.043),  $p = 0.020$ )
- $\text{FeNO}$  and exacerbations ( $r = 0.315$  (95%CI 0.102; 0.500),

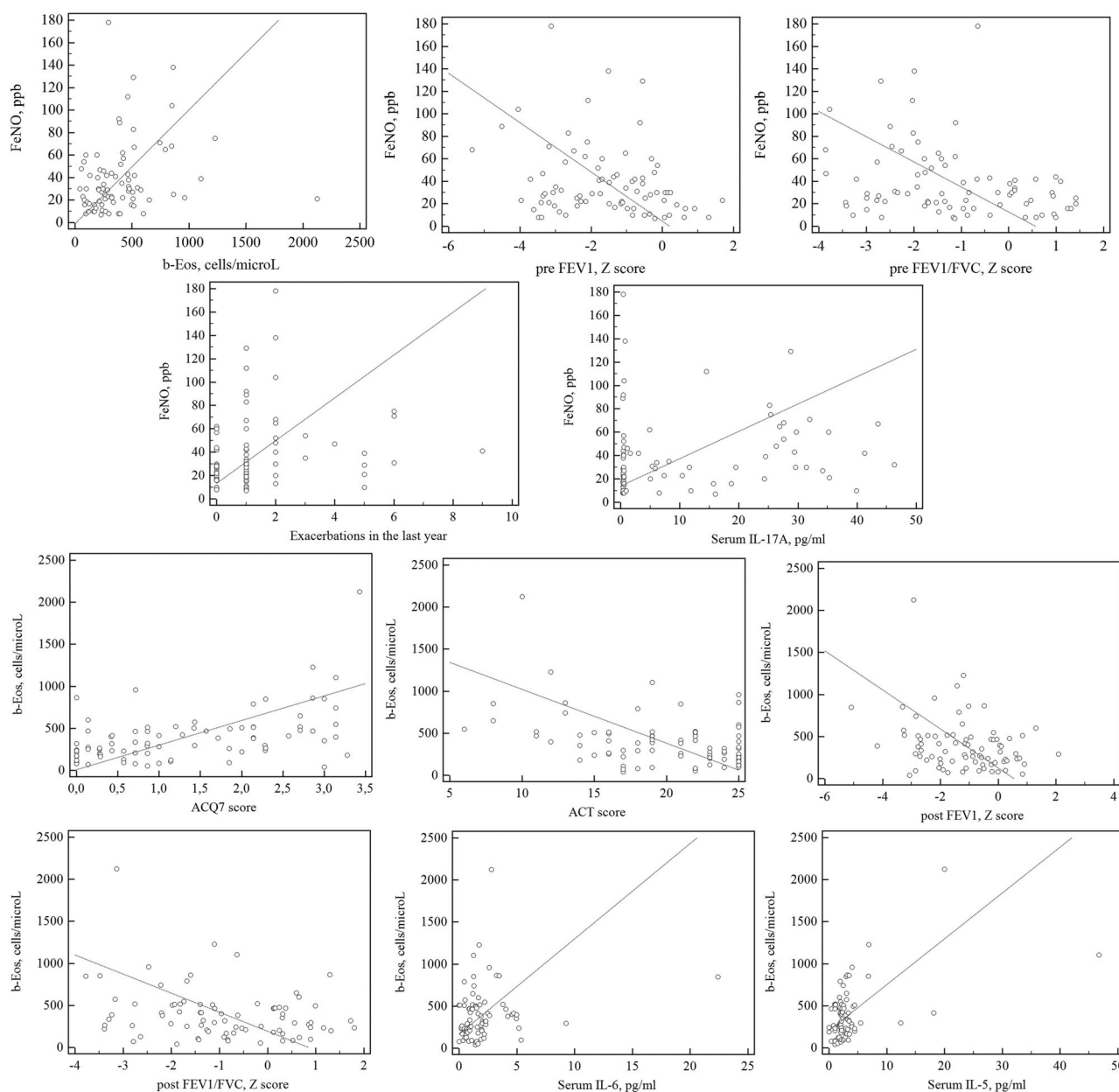


**Fig. 1. Correlations of  $\text{FeNO}$  and b-Eos with other characteristics of the BA Pts.**

Abbreviations: b-Eos – blood eosinophils; ACT – asthma control test; ACQ – asthma control questionnaire;  $\text{FeNO}$  – fraction of exhaled nitric oxide; FVC – forced vital capacity; FEV1 – first second forced expiratory volume; ppb – parts per billion.

- p = 0.004)
- FeNO and serum levels of IL-17A ( $r = 0.241$  (95%CI 0.023; 0.438), p = 0.031)
  - b-Eos and ACQ5 result ( $r = 0.441$  (95%CI 0.247; 0.604), p < 0.001)
  - b-Eos and ACQ7 result ( $r = 0.473$  (95%CI 0.283; 0.628), p < 0.001)
  - b-Eos and ACT result ( $r = -0.334$  (95%CI -0.516; -0.124), p = 0.002)
  - b-Eos and postFEV1 ( $r = -0.259$  (95%CI -0.453; -0.042), p = 0.020)
  - b-Eos and postFEV1/FVC ( $r = -0.224$  (95%CI -0.423; -0.001), p = 0.024)
  - b-Eos and serum levels of IL-5 ( $r = 0.287$  (95%CI 0.07; 0.477), p = 0.010)
  - b-Eos and serum levels of IL-6 ( $r = 0.227$  (95%CI 0.001; 0.426), p = 0.043)
- 3.1. Main clinical, lung functional and inflammatory characteristics of the groups
- The Pts in Group 1 were younger as compared with the other

**Table 2**  
Main characteristics of the BA groups.

|                                       | Group 1<br>↓FeNO/↓b-Eos | Group 2<br>↓FeNO/↑b-Eos | Group 3<br>↑FeNO/↓b-Eos | Group 4<br>↑FeNO/↑b-Eos |
|---------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| N (%)                                 | 23 (28.8)               | 17 (21.3)               | 15 (18.8)               | 25 (31.3)               |
| Age, years <sup>1</sup>               | 37.9 ± 13.9             | 51.5 ± 12.6             | 49 ± 16.3               | 45.8 ± 12.6             |
| Female/male                           | 17/6                    | 9/8                     | 9/6                     | 17/8                    |
| BMI <sup>2</sup>                      | 73.9/26.1%              | 52.9/47.1%              | 60/40%                  | 68/32%                  |
| Smoking history, no/ex/current        | 24.3 (20.3–27.8)        | 29.6 (25.9–33.9)        | 25.7 (22.7–30.3)        | 26.7 (23.7–30.1)        |
| Pack-years                            | 7/5/11                  | 8/5/4                   | 11/3/1                  | 12/10/3                 |
| Onset, age                            | 30.4/21.7/47.8%         | 47.1/29.4/23.5%         | 73.3/20/6.7%            | 48/40/12%               |
| Onset, groups                         | 5 (0–10)                | 4 (0–10)                | 0 (0–2)                 | 3.8 (0–15)              |
| < 20/20–40/> 40                       | 28 (19–38)              | 43 (19–51)              | 25 (7–57)               | 37 (28–49)              |
| SPTs, neg/mono/poly                   | 7/12/4                  | 5/3/9                   | 7/2/6                   | 3/12/10                 |
| Rhinitis, no/yes                      | 30.4/52.2/17.4%         | 29.4/17.6/52.9%         | 46.7/13.3/40%           | 12/48/40%               |
| ACQ5 score <sup>3</sup>               | 5/5/13                  | 4/3/10                  | 2/5/8                   | 8/6/11                  |
| ACQ7 score <sup>4</sup>               | 21.7/21.7/56.5%         | 23.5/17.6/58.8%         | 13.3/33.3/53.3%         | 32/24/44%               |
| ACT score <sup>5</sup>                | 6/17                    | 5/12                    | 3/12                    | 5/20                    |
| Uncontrolled (GINA)                   | 26.1/73.9%              | 29.4/70.6%              | 20/80%                  | 20/80%                  |
| AE/last 1 y <sup>6</sup>              | 0.6 (0–1.6)             | 1 (0.2–2.0)             | 0.2 (0–1.2)             | 2.2 (1.0–3.0)           |
| AE > 1/last y                         | 0.71 (0.14–1.43)        | 1.42 (0.29–2.14)        | 0.29 (0.14–0.86)        | 2.14 (1.10–2.86)        |
| Severe BA (ATS/ERS)                   | 22 (18–25)              | 22 (16–25)              | 23 (17–25)              | 18 (13–21)              |
| Therapy, step 3/4/5                   | 10 (43.5%)              | 7 (41.2%)               | 5 (33.3%)               | 22 (88%)                |
| ICS, high dose                        | 1 (0–1)                 | 0 (0–1)                 | 1 (0–2)                 | 1 (1–2.5)               |
| Use of low dose OCS                   | 4 (17.4%)               | 1 (5.9%)                | 6 (40%)                 | 11 (44%)                |
| Total IgE, IU/ml                      | 6 (26.1%)               | 9 (52.9%)               | 4 (26.7%)               | 16 (64%)                |
| Total IgE > 100 IU/ml                 | 12/11/0                 | 4/12/1                  | 2/10/3                  | 4/15/6                  |
| FeNO, ppb <sup>7</sup>                | 52.2/47.8/0%            | 23.5/70.6/5.9%          | 13.3/66.7/20%           | 16/60/24%               |
| b-Eos, cells/microL <sup>8</sup>      | 6 (26.1%)               | 9 (52.9%)               | 6 (40%)                 | 19 (76%)                |
| b-Neu, 10 <sup>9</sup> L <sup>9</sup> | 0                       | 1 (5.9%)                | 3 (20%)                 | 6 (24%)                 |
| postFVC, Z score                      | 94.9 (13.4–220.5)       | 85.1 (38.9–247.1)       | 102.8(42.9–356)         | 174.7 (65–374.1)        |
| preFEV1/FVC, Z score                  | 11 (47.8%)              | 8 (47.1%)               | 8 (53.3%)               | 17 (68%)                |
| postFEV1/FVC, Z score                 | 16 (10–23)              | 21 (16–24)              | 42 (30–54)              | 62 (40–86)              |
| postFEV1/FVC < LLN                    | 207 (118–260)           | 504 (389–626)           | 200 (94–250)            | 476 (413–767)           |
| preFEV1, Z score                      | 4.0 (3.5–5.6)           | 5.7 (4.7–6.6)           | 3.3 (2.6–5.5)           | 5.0 (4.1–6.2)           |
| postFEV1, Z score                     | −0.39 (−0.88; 0.53)     | −1.15 (−1.54; −0.42)    | −0.17 (−1.12; 0.61)     | −0.77 (−1.51; −0.28)    |
| Change in FEV1, % <sup>*</sup>        | −0.96 (−1.69; 0.47)     | −1.53 (−2.86; 0.47)     | −1.34 (−1.92; 0.12)     | −1.91 (−2.43; −0.54)    |
|                                       | −0.54 (−1.43; 0.56)     | −1.34 (−2.62; 0.46)     | −0.82 (−1.68; 0.24)     | −1.40 (−1.88; −0.045)   |
|                                       | 4 (17.4%)               | 4 (23.5%)               | 2 (13.3%)               | 10 (40%)                |
|                                       | −1.12 (−2.39; 0.20)     | −2.15 (−3.33; −0.41)    | −0.85 (−3.07; −0.16)    | −1.81 (−2.78; −0.87)    |
|                                       | −0.89 (−1.91; 0.25)     | −1.83 (−2.77; −0.37)    | −0.64 (−2.05; 0.20)     | −1.36 (−2.44; −0.37)    |
|                                       | 3 (0–8)                 | 2 (0–12)                | 5 (0–17)                | 8 (4–17)                |

Results are presented as number (%), mean ± standard deviation (*Italic*), median and interquartile range (IQR); The p-value is obtained by Kruskal-Wallis and ANOVA with post hoc analysis.

<sup>1</sup> p < 0.05 group 1 versus 2, 3 and 4.

<sup>2</sup> p < 0.05 group 1 versus 2.

<sup>3</sup> p < 0.05 group 4 versus 1, 2 and 3.

<sup>4</sup> p < 0.05 group 4 versus 1 and 3.

<sup>5</sup> p < 0.05 group 4 versus 1, 2 and 3.

<sup>6</sup> p < 0.05 group 4 versus 1, 2 and group 2 versus 3.

<sup>7</sup> p < 0.05 groups 1 and 2 versus 3 and 4.

<sup>8</sup> p < 0.05 groups 1 and 3 versus 2 and 4.

<sup>9</sup> p < 0.05 group 2 versus 1, 3 and 3 versus 4.

<sup>\*</sup> p < 0.05 group 4 versus 1 and 2.

Abbreviations: AE – acute exacerbations; b-Eos – blood eosinophils; b-Neu – blood neutrophils; OCS – oral corticosteroids; SPTs – skin prick tests; ACT – asthma control test; ACQ – asthma control questionnaire; FeNO – fraction of exhaled nitric oxide; FVC – forced vital capacity; FEV1 – first second forced expiratory volume; GINA – The Global Initiative for Asthma; BMI – body mass index; ATS – American Thoracic Society; ERS – European Respiratory Society; ppb – parts per billion; LLN – Lower Limit of Normal.

Groups. Female gender dominated in all groups, but in Group 2 this difference is not so well presented. Later onset BA was observed in Group 2 and 4. The BMI was higher in Group 2 as compared with Group 1. The percent of current smokers was higher in Group 1 as compared with the other Groups.

The total IgE were similarly elevated in the four groups. Although in Group 4 higher percent of Pts were with abnormal total IgE levels.

Group 4 Pts were with the lowest levels of asthma control and the highest prevalence of severe BA with frequent exacerbations and impaired lung function.

The Pts in Group 2 and 4 were with higher b-Neu in combination of high b-Eos and a trend for worse lung functional parameters. (Table 2).

### 3.2. Interleukins in BA Pts and HC

We have found that the levels of the measured interleukins are significantly higher in Pts with moderate and severe BA as compared with HC (Table 3).

Serum level of IL-5 was significantly higher in Group 2 ( $p < 0.05$  for all comparisons). IL-6 and IL-13 levels were significantly elevated in Group 2, 3 and 4 ( $p < 0.05$  compared with HC). We did not observe statistically significant differences in the levels of IL-8 between the analysed BA Groups and HC. There was no significant difference in the levels of IL-13 between the predefined BA Groups. We have found that IL-17A was significantly elevated in all 4 Groups of BA, as compared with HC ( $p < 0.05$ ). Highest levels of IL-17A were observed in Group 3 and 4 (Table 4 and Fig. 2).

The combined assessment of demographic, clinical, functional, immunological and inflammatory characteristics of the Pts in the different groups is presented by radar charts (Fig. 3). The charts are showing a visualisation of a distinct pattern for each group. The associations between the variables are more clearly presented.

In Group 1 ( $\downarrow$ FeNO/ $\downarrow$ b-Eos) we have observed high prevalence of younger smokers with preserved lung function, normal BMI and less symptomatic. Total IgE was lower in this group. A dominant increase in the average level of IL-8 was seen when compared to the other ILs within the group.

In Group 2 ( $\downarrow$ FeNO/ $\uparrow$ b-Eos), the Pts were older with late onset BA, impaired lung function, high BMI, more symptomatic and severe, with equal elevation of b-Eos and b-Neu, lower FeNO and total IgE. The profile of this group was dominated by elevation in IL-5 and IL-6 levels.

In Group 3 ( $\uparrow$ FeNO/ $\downarrow$ b-Eos) the BA was predominantly with early onset, high FeNO, normal BMI, preserved lung function, less symptomatic, but with more exacerbations. Most of the Pts were non-smokers. b-Eos and b-Neu were equally low. The profile of this group was dominated by elevation in IL-13 in combination with higher IL-17A levels.

In Group 4 ( $\uparrow$ FeNO/ $\uparrow$ b-Eos) were selected highly symptomatic Pts with late onset BA and frequent exacerbations, impaired lung function, high total IgE. The Pts were predominantly non-smokers. The profile of this group was dominated by elevation in IL-17A levels.

**Table 3**  
Serum levels of interleukins in BA Pts and HC.

|        | BA, n = 80 |            |            | HC, n = 30 |           |         | p-value |
|--------|------------|------------|------------|------------|-----------|---------|---------|
|        | median     | IQR        | min-max    | median     | IQR       | min-max |         |
| IL-5   | 2.24       | 1.79-3.28  | 0-46.75    | 1.93       | 0.30-3.10 | 0-5.20  | 0.032   |
| IL-6   | 1.45       | 0.73-2.25  | 0-22.40    | 0.45       | 0.10-1.37 | 0-12.80 | < 0.001 |
| IL-8*  | 1.63       | 0-4.68     | 0-99       | 0.45       | 0-1.70    | 0-10    | 0.047   |
| IL-13  | 1.08       | 0.45-1.89  | 0-157.10   | 0.31       | 0-1.20    | 0-5.40  | 0.014   |
| IL-17A | 1.04       | 0.48-24.45 | 0.38-46.30 | 0.41       | 0.35-0.48 | 0-0.64  | < 0.001 |

Results are presented as median and interquartile range (IQR); Interleukin values are shown as pg/ml; \*n = 70 for BA; The p-value is obtained by Mann-Whitney U test; Abbreviations: IL – interleukin; BA – bronchial asthma; HC – healthy controls; Pts – patients.

## 4. Discussion

This study confirms the heterogeneity of BA especially in real practice, where many factors modify the inflammatory mechanisms of the disease. The distribution of Pts by gender, age, BMI and atopic status was similar to that reported in other studies of adult Pts with severe BA [32,33]. The prevalence of uncontrolled asthma in our study was (46–55%) identical to that reported by Price [34] and Demoly [35]. The incidence of smoking was comparable to that in other real-life studies (about 20–25%) [36,37].

All investigated cytokines in serum were higher in BA as compared with HC. These results demonstrate that moderate and severe BA is associated with persistent inflammatory state as a result of activation of the adaptive and innate immune response.

In 70.1% of the Pts with moderate and severe BA we have found data for high type 2 inflammatory response. Only 28.8% were Pts with type 2-low endotype of BA. The cut-off values for FeNO and b-Eos were determined on the base of previously reported data from other authors.

The cut-off value of b-Eos 300 cells/microL was accepted as appropriate for identification of eosinophilic phenotype of asthma in many studies [38,39]. The median level of b-Eos in our group of Pts with BA is also very near to that cut-off. In the literature are analysed many different FeNO cut-offs depending on the aim of the study (diagnostic ability in corticosteroid naive Pts, management of ICS therapy, use as a biomarker for eosinophilic asthma etc.) and the population (non-smokers, smokers, mild or severe asthma etc.) [40–44]. FeNO cut-offs for detecting eosinophilic asthma varies widely - from 19 ppb to 76 ppb [45,46], but most frequently are used values from 20 to 35 ppb [47,48]. We have decided to use cut-off for FeNO - 30 ppb, because it is in the mentioned range analysed previously and our median FeNO is also near to that value.

According to ATS/ERS recommendations, 43.8% of the Pts were with severe BA and most of them fall into a group with persistent type 2 inflammatory response. The sensitivity of eosinophilic inflammation to corticosteroids is well known, so we are discussing the possibility that Group 1 may include Pts with negative biomarkers for type 2 BA due to a good response to ICS. This leads to a more difficult analysis of the Pts in this group. In group 2 the b-Eos were still high, but the local type 2 inflammation (FeNO level) was suppressed, and this was associated with the lower exacerbations rate. Interestingly, the Pts in groups 4 and 5 were with persistently high FeNO and b-Eos, despite the higher doses of ICS and low maintenance dose of OCS. We suggest that resistance to corticosteroid activity has developed in these Pts.

Pts in Group 1 with low FeNO and b-Eos were predominantly current smokers. In previous studies, smoking was associated with a less eosinophilic inflammation and lower FeNO [31,49,50]. On the other hand, some studies suggest that smoking may sustain the eosinophilic inflammation, particularly in the elderly [50,51]. These observations are consistent with the characteristics of the subjects classified into Group 2 and 4 (high b-Eos), where the Pts are older and some of them were exposed to tobacco smoke in the past. The symptoms, exacerbations and severity were lower in Group 1 as compared with the others

**Table 4**  
Serum levels of interleukins in BA groups.

|           | Group 1 ↓FeNO/↓b-Eos | Group 2 ↓FeNO/↑b-Eos | Group 3 ↑FeNO/↓b-Eos | Group 4 ↑FeNO/↑b-Eos |
|-----------|----------------------|----------------------|----------------------|----------------------|
| N (%)     | 23 (28.8)            | 17 (21.3)            | 15 (18.8)            | 25 (31.3)            |
| IL-5*     | 2.10 (1.75–2.95)     | 3.00 (2.36–4.10)     | 2.05 (1.00–3.31)     | 2.15 (1.48–3.15)     |
| IL-6**    | 0.93 (0.45–1.96)     | 1.79 (1.07–3.02)     | 1.40 (0.60–1.89)     | 1.63 (1.13–3.05)     |
| IL-8      | 2.15 (0.15–4.73)     | 2.58 (0.8–6.9)       | 0.75 (0–7.45)        | 1.70 (0–4.50)        |
| IL-13     | 0.87 (0–1.43)        | 1.11 (0.63–8.00)     | 1.22 (1.00–2.75)     | 1.08 (0.90–1.33)     |
| IL-17A*** | 0.59 (0.42–11.90)    | 0.55 (0.47–5.76)     | 6.08 (0.56–29.60)    | 11.60 (0.51–28.20)   |

Interleukin values are shown as pg/ml. Results are presented as median and interquartile range (IQR); The p-value is obtained by Kruskal-Wallis test with post hoc analysis. IL-8 was measured in n = 70 BA Pts: Group 1 – n = 21; Group 2 – n = 14; Group 3 – n = 13; Group 4 – n = 22.

\*p < 0.05 group 2 versus 1, 3 and 4.

\*\*p < 0.05 group 1 versus 2 and 4.

\*\*\*p < 0.05 groups 3 and 4 versus 1 and 2.

Abbreviations: IL – interleukin; BA – bronchial asthma.

and the lung function despite the smoking is still preserved. The younger age and lower packyears may explain the mismatch in exposure to tobacco smoke and the lung function. The above-mentioned smoking status in Group 1 and the Pts polysensitization to inhaled allergens may lead to their selection in a group of Pts with asthma-COPD overlap in the future. In the radar chart we have found that IL-8 show some tendency of increasement in Group 1. This is consistent with previously reported data for induction of IL-8 from the tobacco smoke [52] and its elevation in plasma of Pts with asthma-COPD overlap [53].

In Group 2 were selected Pts with high b-Eos. The subjects in this group were older with late onset of predominantly severe disease. The gender difference was not so clear, and the BMI was high.

Haldar et al. [54] reported that obesity was associated with increased symptoms, decreased eosinophilic inflammation, female gender, late onset BA and reduced response to ICS. Analysis of Pts participating in The Severe Asthma Research Program (SARP) shows that elevated BMI is associated with specific clinical features in severe asthma, with the identification of a group of Pts who have been associated with female gender, late onset BA and need of systemic corticosteroids [55]. These two studies supported the concept that the asthma phenotype was relatively homogeneous in obese Pts with high symptom expression, low atopy and eosinophilia, and relative corticosteroid resistance, a phenomenon reported also in other clinical trials [56–59].

On other hand Sutherland et al. [60] described 2 different BA obesity clusters. They have found that even in Pts with BMI and BA there was heterogeneity in the expression of symptoms and inflammatory biomarkers. Recent studies have shown that at least two different obese phenotypes can be manifested in BA [61]. Phenotype of allergic BA modulated by the obesity with persistent systemic inflammation and stimulation of Th2 immune response, and another phenotype associated only with the obesity in late-onset non-atopic Pts.

We believe that in Group 2 were included Pts with augmented by the obesity type 2 immune response, resulting in a mixed increasement in both blood eosinophils and neutrophils, and associated with this change in the interleukin profile. We have found association between the b-Eos and serum levels of IL-5 in this group. This result is in agreement with results of other authors [62–64], but in contrast with the results of Akiki et al. [11] They are showing data about cytokine profile composed by IL-5, IL-13, IL-8 and others, that did not show association with the b-Eos, but with b-Neu. Similarly, they are reporting association of this pattern also with obesity. The higher levels of IL-8 may be explained by its key role as chemokine regulating the neutrophil recruitment in this mixed cellular inflammation and the elevation in IL-6 may be a result of the persistent systemic inflammation and metabolic disfunction associated with the obesity in Group 2.

In Group 3 were selected Pts with high FeNO. They were older, but with early onset BA. Most of them were non-smokers with positive SPT to aero-allergens. They were less symptomatic and severe, but more

frequent exacerbators. We confirmed the role of high FeNO as a risk factor for exacerbations in Pts receiving ICS therapy [29,65,66]. Also, in this group we have found a pattern that combines the previously reported data of IL-13 mediated NO production in allergic BA [67–69]. The elevated levels of IL-17A and IL-6 as compared with HC may be associated with the observed frequent exacerbations in this group as it is discussed in other studies [70], and raise the question about their influence in type 2 BA.

Combined elevation of FeNO and b-Eos (Group 4) was associated with the highest prevalence of uncontrolled and severe BA despite the use of high doses ICS and OCS (severe refractory to therapy BA). These data are consistent with results reported by other authors for association of these two biomarkers with the BA severity [39,42,65,71,72]. In Group 4 were predominantly non-smoker females with late onset BA, positive to aero-allergens, with high prevalence of rhinitis and elevated total IgE. The higher levels of IL-6, IL-13 and IL-17A in Group 4 are reflecting the combined activation of the immune system in Pts with refractory to therapy type 2 severe BA and underline the need of further investigation of this group for development of more personalised approach. Our study supports the idea for IL-17A enhancement of Th2 mediated immune response in BA. The combination of type 2/IL-17A was associated with more severe, uncontrolled and refractory BA.

The levels of IL-17A in serum are discussed as an independent risk factor for the development of severe BA [73]. Th17 activity results in increased airway smooth muscle contractility and proliferation and is associated more often with corticosteroid-resistant and neutrophilic BA [74–77]. Agache and Akdis [24] are presenting an idea for mixed type2/Th17 endotype. They suggest that type 2 and IL-17 immune response may cause excessive tissue damage. On other hand Th2/Th17 cells are identified [78] in BAL fluid of BA Pts with corticosteroid resistance and airway obstruction [79]. Seys et al. [9] are describing an “IL-5, IL-17A, IL-25-high” airway inflammatory pattern of uncontrolled adult BA despite the maintenance therapy. The results of Hasegawa et al. [10] are also showing upregulation of IL-17A in association with type 2 cytokines in serum of uncontrolled BA Pts.

The heterogeneity of the studied population gave us the opportunity to evaluate the ability of the type 2 (b-Eos and FeNO) biomarkers for identification of more severe BA groups in the real clinical practice. We have found that moderate and severe BA subgroups demonstrate different serum interleukin profile. The identification of the type 2/IL-17A pattern described in our study may be used for better stratification of the Pts with severe, refractory BA in the development of new therapies.

Limitation of our study was the absence of longitudinal period for confirmation of the stability of these patterns. Future extension of this study, including a BAL or sputum analysis, will contribute to a deeper understanding of the characteristics of these groups.

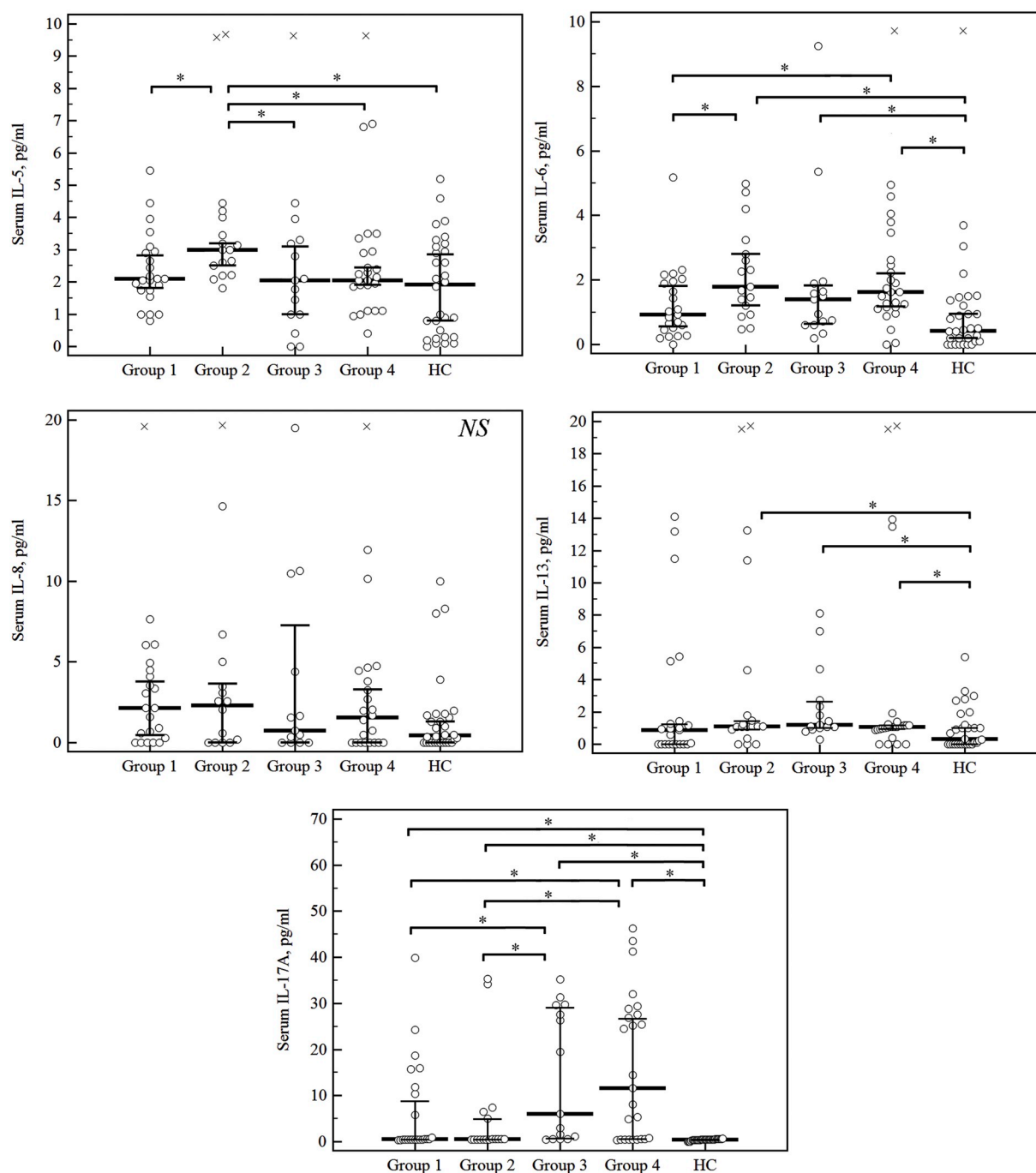


**Fig. 2.** Intergroup comparisons of serum interleukin levels.

The p-value was obtained by Kruskal-Wallis post hoc analysis and Bonferroni correction. \*

p <

0.05; · – outlier; IL-8 was measured in n = 70 BA

Pts: Group 1 – n =

21; Group 2 – n =

14; Group 3 – n =

13; Group 4 – n =

22;

Abbreviations: Pts – patients; HC – healthy controls; NS – not significant; Error bars: 95%CI for median.

## 5. Conclusions

Combined measurement of FeNO and b-Eos is useful in the identification of severe type 2 BA subgroups with frequent exacerbations. IL-5, IL-6, IL-13 and IL-17A are involved in the persistent type 2 immune

response in moderate and severe BA. We have identified a pattern of refractory, severe type 2/IL-17A high BA. Our results support and expand the knowledge of moderate and severe BA in the real clinical practice.

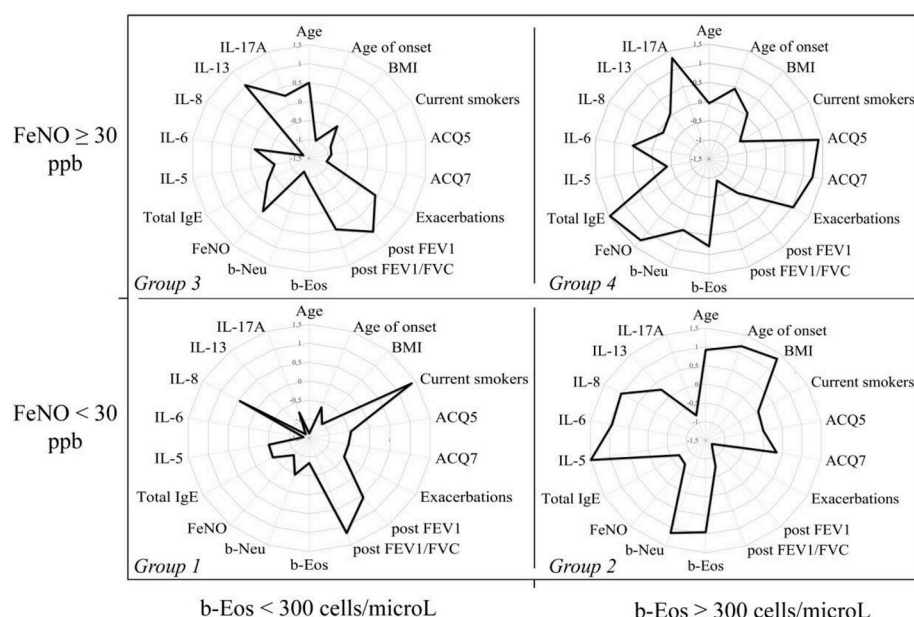


**Fig. 3.** The radar charts. Radar charts were used for better visualisation of the group characteristics in association with the serum levels of the measured interleukins. From the centre to the periphery of the chart, the variable value is increasing. The scale from  $-1.5$  to  $+1.5$  represent the z score for each measurement.

Abbreviations: ACQ - Asthma Control Questionnaire; BMI - Body Mass Index; b-Eos - Absolute Blood Eosinophilic Count; b-Neu - Absolute Blood Neutrophilic Count; FeNO - Fraction of Exhaled Nitric Oxide; FEV1 - First Second Forced Expiratory Volume; FVC - Forced Vital Capacity; IL - Interleukin.

## Acknowledgments of funding

The research was funded by GRANT project ID 308/2015, contract №75/2015 to Council of Medical Science, Medical University Sofia, Bulgaria

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