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Blood eosinophils, FeNO and small airways dysfunction in predicting airway hyperresponsiveness in patients with asthma-like symptoms

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ABSTRACT

Purpose: In patients with suspected asthma and no airflow limitation in spirometry, methacholine challenge testing (MCT) for airway hyperresponsiveness (AHR) is an option of documenting variable airflow limitation. The goal of the study was to assess the ability of blood eosinophils, fractional concentration of exhaled nitric oxide (FeNO) and distal airways function to discriminate patients with AHR from those with normal airway responsiveness (AR).

Methods: We analyzed baseline data from 42 participants who underwent MCT because of asthma-like symptoms and no airflow limitation in spirometry.

Results: Eosinophil count was higher among participants with borderline AHR comparing to those with normal AR (340 cells/ μ L, IQR 285-995 vs. 125 cells/ μ L, IQR 75-180, post-hoc $p=0.041$). FeNO and percent predicted of functional residual volume (FRC%pred) were higher in participants with moderate-marked AHR compared to those with normal AR (40 ppb, IQR 30.5-100.5 vs. 18 ppb, IQR 13-50, post-hoc $p=0.008$; $140.1\pm17.0\%$ vs. $107.3\pm20.7\%$, post-hoc $p<0.001$, respectively). Percentage predicted of the maximal expiratory flow at 25% of the forced vital capacity (MEF₂₅%pred) was lower in participants with mild AHR and borderline AHR compared to those with normal AR ($72.9\pm16.9\%$ vs. $113.0\pm36.8\%$, post-hoc $p=0.017$; $73.3\pm15.9\%$ vs. $113.0\pm36.8\%$, post-hoc $p=0.045$; respectively). Level of AHR correlated with eosinophil count, FeNO, MEF₂₅%pred, forced expiratory flow between 25% and 75% of vital capacity (FEF₂₅₋₇₅%pred), FRC%pred and specific airway resistance (sR_{aw}).

Conclusions: Blood eosinophils, FeNO and small airways dysfunction markers are related to the level of AR to methacholine in patients with asthma-like symptoms and no airflow limitation in spirometry.

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Introduction

Asthma is the most common chronic disease among children and young adults (1). Diagnosis of asthma is based on the presence of characteristic symptoms, such as wheezing, shortness of breath, chest tightness or cough and variable expiratory airflow limitation (2,3). The diagnosis is usually obvious in patients with typical asthma-like symptoms and airflow limitation with reversibility in spirometry (4). However, patients with asthma may demonstrate normal spirometry and previously reported sensitivity of reduced forced expiratory volume in 1 s (FEV₁) in detecting asthma is low (16%-29%) (5,6). Absence of airflow limitation in spirometry is common in mild asthma and among patients who are asymptomatic at the time of evaluation (7). However, normal FEV₁, forced vital capacity (FVC), FEV₁/FVC and FEV₁/VC ratios may be

observed even in patients with active and symptomatic asthma, especially in the early stages of disease (5,8).

In patients with clinical suspicion of asthma and normal FEV₁/(F)VC ratio evaluation of airway hyperresponsiveness (AHR) is an option of documenting variable expiratory airflow limitation, which favors asthma diagnosis (2). AHR is one of the key asthma features and is usually assessed by methacholine challenge testing (MCT) (9). Airway response to methacholine is assessed on the basis of percentage decrease in FEV₁ after inhalation of methacholine solutions (10,11). MCT is highly sensitive in excluding current asthma in symptomatic patients (10,12,13). The result of MCT is negative and effectively excludes current asthma in patients with asthma-like symptoms, if provocative concentration or dose of methacholine causing a 20% fall in FEV₁ (PC₂₀ or PD₂₀) is >16 mg/

mL or $>400\mu\text{g}$, respectively (10,11). Borderline AHR is diagnosed if PC_{20} or PD_{20} are within 4–16 mg/mL or 100–400 μg , respectively (10,11). The result of MCT is considered positive when PC_{20} or PD_{20} are $\leq 4\text{ mg/mL}$ or $\leq 100\mu\text{g}$, respectively (10,11).

FEV_1 reflects mainly large airway function and baseline FEV_1 may be less sensitive in diagnosing patients with dominant small airway inflammation, which is a component of asthma pathomechanism (14,15). Function of small airway is reported to be better reflected by airway resistance parameters measured by forced oscillation technique (FOT), plethysmographic airway resistance measures, functional residual capacity measured by plethysmography ($\text{FRC}_{\text{pleth}}$) as well as nonstandard spirometric measures, including mid expiratory flow rates and forced expiratory flow between 25% and 75% of vital capacity (FEF_{25-75}) (8,16–21). Airway resistance parameters measured by FOT and plethysmography, as well as FEF_{25-75} were previously postulated to predict the presence of AHR (8,22–24). $\text{FRC}_{\text{pleth}}$ was also reported previously to correlate significantly with the severity of distal lung eosinophilic inflammation (25). Furthermore, blood eosinophil count and the fractional concentration of exhaled nitric oxide (FeNO) were shown to be associated with AHR (26–28).

It is crucial to document the diagnosis of asthma at initial evaluation, before starting controller treatment (2). Characteristic clinical features as well as pulmonary function abnormalities may improve spontaneously or upon commencement of the controller treatment (2). In this context, it should be emphasized, that MCT is not widely accessible, especially in the setting of primary or secondary care clinics. Furthermore, repeated FEV_1 measurement during MCT impose significant burden on examined patients. The procedure is also time-consuming and problematic both for patients and pulmonary function laboratory technicians. A negative MCT lasts about one hour (29). Thus, there is an unmet need for easy to perform, quick, cost-effective and accessible methods of evaluation of patients presenting asthma-like symptoms with no airflow limitation in baseline spirometry.

In an earlier publication on the same cohort we reported acceptable performance of airway resistance measures as the alternative read-out for airway obstruction during MCT and we showed that the onset of symptoms during MCT is better reflected by changes in airway resistance measures than by FEV_1 decrease (30). The goal of the present study was to assess the ability of blood eosinophils, FeNO and dysfunction of distal airways to discriminate patients with AHR from those with normal airway responsiveness.

Methods

Study design

We performed a cross-sectional study in patients with asthma-like symptoms, who underwent MCT in our lung function laboratory according to the protocol based on American Thoracic Society (ATS) guidelines (11), modified by adding the assessment of airway resistance parameters and $\text{FRC}_{\text{pleth}}$ to the measurement of FEV_1 at each step of MCT. We analyzed patients demographic data, clinical symptoms, eosinophilic count as well as pretest pulmonary function measures in relation to the level of airway responsiveness to methacholine.

Participants

We analyzed data from 42 participants (21 women/21 men, median age 36 years, IQR 30–56). Baseline assessment included detailed medical history, spirometry and airway resistance measurement using FOT, plethysmography and interrupter technique as well as $\text{FRC}_{\text{pleth}}$ measurement. All of included participants reported asthma-like symptoms (cough, shortness of breath, wheezing or chest tightness) and had no airflow limitation, defined on the basis of decreased FEV_1/VC ratio below the 5th percentile. Study participants underwent MCT because of clinical suspicion of asthma and none of them was diagnosed with any other chronic lung disease.

The distribution of the level of airway responsiveness (AR) to methacholine among study participants was as follows: normal AR ($\text{PC}_{20} >16\text{ mg/mL}$) in 17 participants (40.5%), borderline AHR (PC_{20} of 4–16 mg/mL) in 5 participants (11.9%), mild AHR ($1\text{ mg/mL} < \text{PC}_{20} \leq 4\text{ mg/mL}$) in 7 participants (16.7%), moderate AHR ($0.25\text{ mg/mL} < \text{PC}_{20} \leq 1\text{ mg/mL}$) in 5 patients (11.9%) and marked AHR ($\text{PC}_{20} \leq 0.25\text{ mg/mL}$) in 8 participants (19%). Detailed characteristics of participants was published previously (30).

All participants gave written informed consent prior to their participation in the trial. Study protocol was approved by the Medical University of Warsaw Bioethics Board and was registered at ClinicalTrials.gov – NCT02343419.

Spirometry

Spirometric measures were performed by LungTest 1000 spirometer (MES, Cracow, Poland) according to the ATS/ERS recommendations (31). We recorded FEV_1 , forced expiratory volume in 6 s (FEV_6) forced vital capacity (FVC) as well as measures previously reported to reflect the function of small airways: the

maximal expiratory flow at 25%, 50% and 75% of the forced vital capacity (MEF_{25} , MEF_{50} , MEF_{75} , respectively), forced expiratory flow between 25% and 75% of forced vital capacity (FEF_{25-75}) (16,17,20,32).

Blood eosinophils, FeNO

We included in the analysis the result of blood eosinophilic count performed within 2 weeks prior to the MCT. FeNO was measured at exhalation flow rate of 50 ml/s using a FeNO+ equipment (Medisoft, Sorinnes, Belgium) according to ATS/ERS guidelines (33). Measurement was performed directly before MCT.

Forced oscillation technique, plethysmography, interrupter technique

Forced oscillation airway resistance (R_{FOT}) and reactance (X_{FOT}) were measured with the use of Micro 5000 Rosc equipment (Medisoft, Sorinnes, Belgium) according to the ERS Task Force guidelines (34). Forced oscillation airway resistance measured at 5 Hz (R_{FOT_5}), 10 Hz ($R_{FOT_{10}}$) as well as the difference in resistance between 5 Hz and 20 Hz ($R_{FOT_{5-20}}$) were included in the final analysis. Plethysmographic airway resistance measures as well as thoracic gas volume (TGV) at the end of expiration, i.e. FRC_{pleth} were measured using BodyBox 5500 cabin plethysmograph (Medisoft, Sorinnes, Belgium) according to the principles described in ERS Monograph (35). Interrupter resistance (R_{INT}) was measured using dedicated module of BodyBox 5500 plethysmograph (Medisoft, Sorinnes, Belgium) according to the ERS Task Force recommendations (36).

Methacholine challenge testing

MCT was performed according to the protocol based on American Thoracic Society (ATS) guidelines (11), modified by adding the assessment of airway resistance parameters by FOT, plethysmography and interrupter technique to the routine measurement of FEV_1 . The exact details of the protocol were published previously (30).

Statistical methods

We present continuous variables with normal distribution as mean values $\pm$ standard deviations (SD), whereas those non-normally distributed as medians and interquartile ranges (IQR). We used the independent Student's t-test to assess differences between

two groups in normally distributed variables with homogenous variances and Welch's t-test to assess differences between two groups in normally distributed variables with non-homogenous variances. For non-normally distributed variables, we used Mann-Whitney U test to assess differences between two groups and Kruskal-Wallis test to assess differences between ≥ 3 groups. Differences in categorical variables between two groups were assessed using the Fisher's exact test and between ≥ 3 groups using the Pearson's chi-squared test. Fisher's exact approach was also used for *post hoc* analysis of a Pearson's chi-squared test (37). The Benjamini-Hochberg adjustment procedure with the false discovery rate set at 10% was used to correct for multiple testing. We used receiver operating characteristic (ROC) curves for the assessment of the diagnostic yield of baseline pulmonary function measures in the diagnosis of bronchial hyperresponsiveness to methacholine. Correlations between variables were assessed using Spearman rank correlation coefficient. Multiple logistic regression analysis was used to determine factors associated with the results of MCT. Calculations were performed using Statistica data analysis software system, version 13 (TIBCO Software Inc., 2017, <http://statistica.io>). *P* values of <0.05 was considered statistically significant.

Results

Baseline measures in relation to the level of airways responsiveness

In Table 1 we present demographic data, smoking status, clinical symptoms, blood eosinophils and FeNO of subjects with different levels of airway responsiveness to methacholine. We observed significant differences in BMI ($p=0.029$), smoking status ($p=0.043$), frequency of patient-reported dyspnea ($p=0.007$) as well as eosinophil count ($p=0.008$) and FeNO ($p=0.016$) across different levels of AR. In post-hoc analysis we observed that there were more smokers among patients with borderline AHR compared to those with normal AR [0 out of 17 (0%) vs. 2 out of 5 (40%), post-hoc $p=0.043$]. Furthermore, patients with moderate-marked AHR reported dyspnea significantly more frequently compared to those with both borderline AHR [11 out of 13 (84.6%) vs. 1 out of 5 (20%), post-hoc $p=0.022$] and normal AR [11 out of 13 (84.6%) vs. 5 out of 17 (29.4%), post-hoc $p=0.004$]. We also observed that eosinophil count was significantly higher among patients with borderline AHR comparing to those with normal AR (340 cells/ μ L,

Table 1. Demographic data, smoking status, symptoms, blood eosinophils and FeNO in subjects with different levels of airway responsiveness to methacholine.

	Moderate-marked AHR $PC_{20} \leq 1$ mg/mL, $n = 13$	Mild AHR 1 mg/ mL < $PC_{20} \leq 4$ mg/mL, $n = 7$	Borderline AHR 4 mg/ mL < $PC_{20} \leq 16$ mg/mL, $n = 5$	Normal AR $PC_{20} > 16$ mg/ mL, $n = 17$	p
Males, n (%)	6 (46.2%)	3 (42.9%)	1 (20%)	11 (64.7%)	0.322
Age, years	37 (33–54)	31 (24–32)	56 (47–58)	36 (30–63)	0.097
Height, cm	171.8 (± 9.3)	172.9 (± 5.3)	167.4 (± 2.8)	171.2 (± 11.6)	0.780
BMI, kg/cm ²	24.4 (± 4.2)	23.2 (± 2.6)	28.8 (± 3.1)	27.0 (± 3.9)	0.029*
Smokers, n (%)	1 (7.7%)	2 (28.6%)	2 (40%) [†]	0 (0%) [†]	0.043
Cough	8 (61.5%)	6 (85.7%)	5 (100%)	13 (76.5%)	0.327
Dyspnea	11 (84.6%) ^{‡,§}	5 (71.4%)	1 (20%) [‡]	5 (29.4%) [§]	0.007
Wheezing	9 (69.2%)	4 (57.1%)	2 (40.0%)	4 (23.5%)	0.082
Chest tightness	5 (38.5%)	3 (42.9%)	0 (0%)	7 (41.2%)	0.362
Eosinophils, cells/ μ L	290 (160–430)	160 (50–250)	340 (285–995) [†]	125 (75–180) [†]	0.008
FeNO, ppb	40 (30.5–100.5) [†]	24 (16–38)	18 (13–50)	18 (10–30) [†]	0.016

*Significant global effect with non-significant post-hoc tests.

^{†,‡,§,†}Significant difference in post-hoc tests between patients with different levels of airway responsiveness to methacholine.

Significant p values are boldtyped.

IQR 285–995 vs. 125 cells/ μ L, IQR 75–180, post-hoc $p = 0.041$). Finally, FeNO was significantly higher in subjects with moderate-marked AHR compared to those with normal AR to methacholine (40 ppb, IQR 30.5–100.5 vs. 18 ppb, IQR 13–50, post-hoc $p = 0.008$). In Figure 1 we present differences in eosinophil count and FeNO across different levels of AR to methacholine.

In Table 2 we present the comparison of pulmonary function parameters measured by spirometry, plethysmography, FOT and interrupter technique in participants with different level of AR to methacholine. We observed significant differences in $MEF_{25}\%$ pred ($p = 0.006$) and $FRC_{pleth}\%$ pred ($p < 0.001$) across different levels of AR to methacholine (Figure 2). We found that MEF_{25} expressed as the percentage of predicted was significantly lower in subjects with mild AHR and

borderline AHR compared to those with normal AR ($72.9\% \pm 16.9\%$ vs. $113.0\% \pm 36.8\%$, post-hoc $p = 0.017$; $73.3\% \pm 15.9\%$ vs. $113.0\% \pm 36.8\%$, post-hoc $p = 0.045$; respectively). We also recorded that $FRC_{pleth}\%$ pred was significantly higher in subjects with moderate-marked AHR compared to those with normal AR ($140.1\% \pm 17.0\%$ vs. $107.3\% \pm 20.7\%$, post-hoc $p < 0.001$).

We did not find significant differences in baseline airway resistance parameters measured by FOT, plethysmography and interrupter technique between participants with different levels of AR to methacholine.

Correlation between baseline parameters and the level of AR

We observed that level of airway responsiveness to methacholine, ranked as (1) normal AR, (2) borderline AHR, (3) mild AHR, (4) moderate and (5) marked AHR, correlated significantly with eosinophil count, FeNO, $MEF_{25}\%$ pred, $FEF_{25-75}\%$ pred, $FRC_{pleth}\%$ pred and sR_{aw} . Baseline measures, which correlates significantly with the level of AHR graded on a 5-point scale: (0-Normal AR; 1-Borderline; 2-Mild; 3-Moderate; 4-Marked AHR) are presented in descending order in Table 3. Scatterplots presenting correlations between of the AHR level with $FRC_{pleth}\%$ pred ($R = 0.61$) and FeNO ($R = 0.50$) are shown in Figure 3.

Baseline measures in predicting airway hyperresponsiveness

In Table 4 we present areas under ROC curves of selected baseline measures for identifying subjects with borderline to marked ($PC_{20} \leq 16$ mg/mL) and mild to marked ($PC_{20} \leq 4$ mg/mL) airway responsiveness to methacholine. For discriminating subjects with $PC_{20} \leq 16$ mg/mL from those with > 16 mg/mL we observed

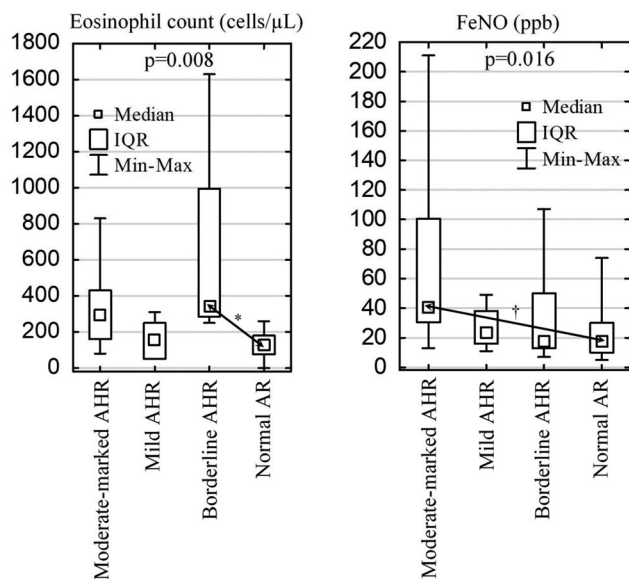
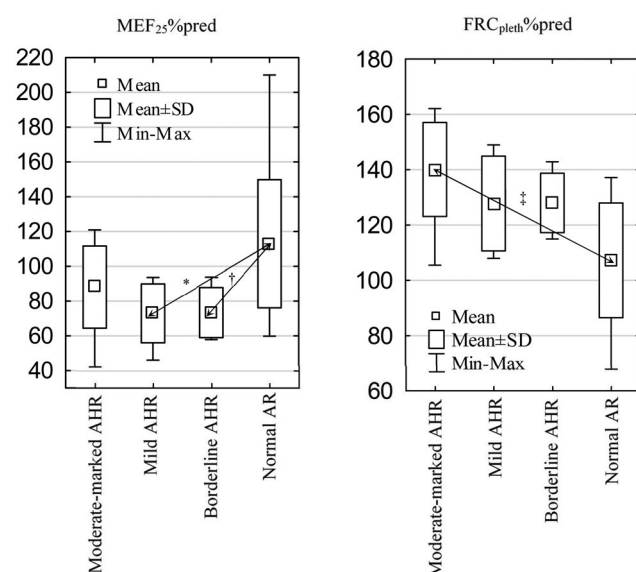
**Figure 1.** Eosinophil count and fractional concentration of exhaled nitric oxide in patients with different levels of airway responsiveness to methacholine.

Table 2. Comparison of baseline pulmonary function measures between participants with different levels of airway responsiveness to methacholine.

	Moderate-marked AHR $PC_{20} \leq 1$ mg/mL, $n = 13$	Mild AHR 1 mg/ $mL < PC_{20} \leq 4$ mg/mL, $n = 7$	Borderline AHR 4 mg/ $mL < PC_{20} \leq 16$ mg/mL, $n = 5$	Normal AR $PC_{20} > 16$ mg/ mL, $n = 17$	p
FEV ₁ %pred, %	95.3 (± 16.1)	88.5 (± 8.3)	86.3 (± 18.4)	97.9 (± 10.8)	0.236
FEV ₁ /FVC, %	77.0 (± 6.8)	77.9 (± 5.3)	74.0 (± 5.4)	77.5 (± 5.3)	0.395
FEV ₆ /FVC, %	99.4 (98.3–99.5)	99.7 (98.6–99.8)	97.4 (94.0–97.7)	99.0 (98.4–99.4)	0.556
MEF ₅₀ %pred, %	77.1 (± 19.9)	78.3 (± 19.2)	74.2 (± 26.1)	91.5 (± 23.5)	0.223
MEF ₂₅ %pred, %	88.0 (± 23.6)	72.9 (± 16.9)*	73.3 (± 14.4) [†]	113.0 (± 36.8)* [†]	0.006
FEF _{25–75} %pred, %	79.7 (± 19.7)	77.2 (± 17.1)	74.3 (± 15.9)	98.6 (± 28.0)	0.052
R _{aw} kPa/L/s	0.19 (± 0.05)	0.17 (± 0.07)	0.17 (± 0.06)	0.18 (± 0.05)	0.891
sR _{aw} kPa-s	0.81 (± 0.25)	0.68 (± 0.25)	0.61 (± 0.22)	0.61 (± 0.26)	0.176
FRC _{pleth} %pred, %	140.1 (± 17.0) [†]	127.8 (± 17.1)	128.0 (± 10.7)	107.3 (± 20.7) [†]	<0.001
R _{FOT-5} kPa/L/s	1.27 (0.91–1.56)	1.12 (0.87–1.33)	0.74 (0.65–0.96)	1.16 (0.93–1.31)	0.254
R _{FOT-10} kPa/L/s	0.47 (0.43–0.52)	0.43 (0.39–0.47)	0.44 (0.44–0.44)	0.45 (0.44–0.50)	0.245
R _{FOT-5–20} kPa/L/s	0.85 (0.51–1.11)	0.73 (0.50–0.95)	0.29 (0.29–0.46)	0.73 (0.62–0.98)	0.151
R _{INT} kPa/L/s	0.36 (0.30–0.42)	0.32 (0.25–0.40)	0.42 (0.34–0.46)	0.32 (0.27–0.4)	0.499

*†: Significant difference in post-hoc tests between patients with different levels of airway responsiveness to methacholine.

Significant p values are boldtipped.

**Figure 2.** Spirometric flow rates and plethysmography-derived functional residual capacity in patients with different level of airway responsiveness to methacholine.**Table 3.** Significant correlations between baseline measures and the level of airway responsiveness to methacholine.

Baseline measures	Correlation with the level of AHR	
	R	p
Eosinophil count	0.40	0.033
FeNO	0.50	0.001
MEF ₂₅ %pred	−0.36	0.019
FEF _{25–75} %pred	−0.33	0.035
FRC _{pleth} %pred	0.61	<0.001
sR _{aw}	0.33	0.033

AUC significantly different from 0.5 for the following baseline measures: (1) eosinophil count; (2) FeNO; (3) MEF₂₅%pred; (4) FEF_{25–75}%pred; (5) FRC_{pleth}%pred. For discriminating participants with $PC_{20} \leq 4$ mg/mL from those with $PC_{20} > 4$ mg/mL, we observed AUC significantly different from 0.5 for following baseline measures: (1) FeNO; (2) MEF₂₅%pred; (3)

FRC_{pleth}%pred; (4) sR_{aw}. For discriminating subjects with $PC_{20} \leq 1$ mg/mL from those with $PC_{20} > 1$ mg/mL AUC was significantly different from 0.5 for following measures: (1) eosinophil count; (2) FeNO; (3) FRC_{pleth}%pred; (4) sR_{aw}.

Figure 4 shows ROC curves for the baseline measures with the highest diagnostic accuracy in discriminating subjects with different levels of AR to methacholine. Table 5 shows optimal cutoff values of baseline measures with AUC significantly different from 0.5 for discriminating patients with different levels of AR to methacholine.

We used logistic regression analysis to assess the relationship between the result of MCT and following covariates: BMI, FeNO, FRC_{pleth}%pred, MEF₂₅%pred (Table 6). In the results of the logistic regression model, we showed that lower MEF₂₅%pred and higher FRC_{pleth}%pred were independent predictors of the positive result of MCT ($PC_{20} \leq 16$ mg/mL), whereas BMI and FeNO were not significant. When combining above variables into the logistic model for the prediction of the mild-marked AHR ($PC_{20} \leq 4$ mg/mL), we showed that lower BMI and higher FRC_{pleth}%pred were independent predictors, whereas MEF₂₅%pred and FeNO were not significant. Finally, only higher FRC_{pleth}%pred was an independent predictor of moderate-marked AHR. The discrimination ability for above models was good to excellent (AUC = 0.95, AUC = 0.89 and AUC = 0.85, respectively).

Discussion

We demonstrated that the presence of AHR among patients with asthma-like symptoms and no airflow limitation in baseline spirometry is related to the increased number of blood eosinophils, FeNO and FRC_{pleth} as well as to decreased mid expiratory flow

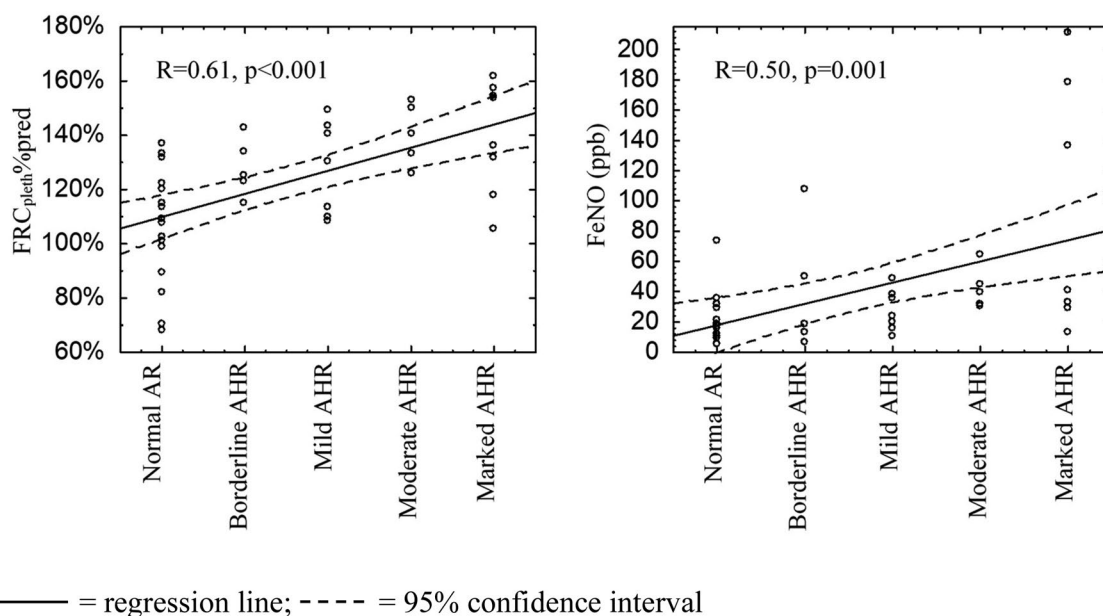


Figure 3. Correlation of the airway hyperresponsiveness level with percent predicted of functional residual capacity measured and fractional concentration of exhaled nitric oxide.

Table 4. Areas under the receiver operating characteristic curves of selected baseline measures for identifying subjects with different levels of airway responsiveness to methacholine.

	PC ₂₀ ≤16 mg/mL vs. PC ₂₀ >16 mg/mL		PC ₂₀ ≤4 mg/mL vs. PC ₂₀ >4 mg/mL		PC ₂₀ ≤1 mg/mL vs. PC ₂₀ > 1 mg/mL	
	AUC (95% CI)	<i>p</i>	AUC (95% CI)	<i>p</i>	AUC (95% CI)	<i>p</i>
Eosinophil count	0.80 (0.63–0.96)	<0.001	0.59 (0.38–0.81)	0.409	0.73 (0.54–0.92)	0.019
FeNO	0.75 (0.59–0.90)	0.002	0.75 (0.60–0.90)	0.001	0.80 (0.66–0.94)	<0.001
MEF ₂₅ %pred	0.80 (0.65–0.94)	<0.001	0.68 (0.51–0.84)	0.035	0.54 (0.35–0.72)	0.706
FEF _{25–75} %pred	0.74 (0.57–0.91)	0.006	0.66 (0.50–0.83)	0.056	0.62 (0.44–0.80)	0.192
FRC _{pleth} %pred	0.85 (0.73–0.96)	<0.001	0.80 (0.67–0.94)	<0.001	0.82 (0.68–0.96)	0.000
sR _{aw}	0.64 (0.46–0.82)	0.118	0.68 (0.51–0.84)	0.038	0.71 (0.55–0.87)	0.012

Significant *p* values are boldtyped.

parameters. Increased FRC_{pleth} and decreased MEF₂₅%pred were demonstrated to effectively predict borderline-marked AHR. Decreased BMI and increased FRC_{pleth}%pred were demonstrated to effectively predict mild-marked AHR, whereas moderate-marked AHR was predicted by increased FRC_{pleth}%pred.

We performed multi-approach baseline assessment of patients with asthma symptoms and normal FEV₁, FEV₁/(F)VC ratio in spirometry. We aimed to find baseline markers, which are related to the presence of AHR in such patients and may increase or decrease the probability of asthma diagnosis without the need for bronchoprovocation. To the best of our knowledge, this is the first study which included assessment of baseline airway function by spirometry, FOT, plethysmography, interrupter technique and measurement of FeNO as well as MCT in non-obstructive patients suspected of asthma.

Our results indicate that multi-approach baseline assessment of patients with asthma-like symptoms and no airflow limitation in spirometry may provide

information regarding the probability of the AHR presence without performing burdensome and poorly available airway challenge. We demonstrated that impaired small airway function and airway inflammation parameters are related to the AHR among patients with asthma-like symptoms and no airflow limitation in spirometry. In symptomatic patients with normal “classic” parameters of airflow limitation in spirometry, the presence of AHR and respiratory symptoms may be related to the inflammation with the predilection for more peripheral airway regions. Recording elevated blood eosinophils and FeNO indicates high probability of current airway inflammation, whereas recording increased FRC_{pleth} and decreased mid expiratory flow parameters provides the proof for distal airway dysfunction. We observed that both easily accessible measures, i.e. spirometric mid expiratory flow parameters and eosinophils count, as well as more sophisticated plethysmographic examination and FeNO measurement were able to detect baseline abnormalities in patients who tested positive for AHR.

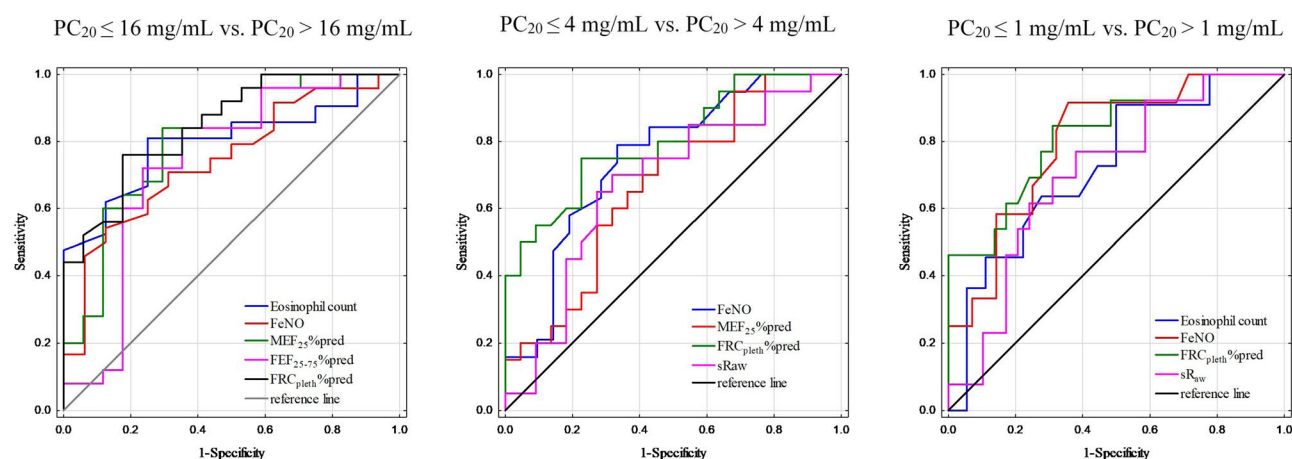


Figure 4. Receiver-operating characteristics (ROC) curves for the baseline measures with the highest diagnostic accuracy for identifying subjects with different levels of airway responsiveness to methacholine.

Table 5. Optimal cutoff values of baseline measures for identifying patients with different levels of airway responsiveness to methacholine.

Baseline measures	Cutoff value	Sensitivity	Specificity	LR ⁺	LR ⁻	YI
PC₂₀ ≤ 16 mg/ml vs. PC₂₀ > 16 mg/ml						
Eosinophil count	150 cells/μL	81%	75%	3.2	0.25	0.56
FeNO	24 ppb	70.0%	77.3%	2.27	0.42	0.40
MEF ₂₅ %pred	95.6%	84%	70.6%	2.9	0.23	0.55
FEF ₂₅₋₇₅ %pred	92.9%	84%	64.7%	2.4	0.25	0.49
FRC _{pleth} %pred	122.8%	76%	82.4%	4.3	0.29	0.58
PC₂₀ ≤ 4 mg/ml vs. PC₂₀ > 4 mg/ml						
FeNO	24 ppb	78.9%	66.7%	2.1	0.32	0.46
MEF ₂₅ %pred	95.6%	80%	54.5%	1.76	0.37	0.35
FRC _{pleth} %pred	126.0%	75%	77.3%	3.3	0.32	0.52
sR _{aw}	0.70 kPa·s	65%	72.7%	2.4	0.48	0.38
PC₂₀ ≤ 1 mg/ml vs. PC₂₀ > 1 mg/ml						
Eosinophil count	150 cells/μL	90.9%	50.0%	1.8	0.18	0.41
FeNO	39 ppb	58.3%	85.7%	4.1	0.49	0.44
FRC _{pleth} %pred	150%	46.2%	100%	n/a	0.54	0.46
sR _{aw}	0.81 kPa·s	46.2%	82.8%	2.7	0.65	0.29

Table 6. Logistic regression model for the prediction of the result of methacholine challenge test.

A. Prediction of borderline-marked AHR (PC ₂₀ ≤ 16 mg/mL)			
Variables	OR	95% CI	p-value
BMI	0.91	0.66–1.26	0.578
FeNO	1.06	0.98–1.15	0.120
FRC _{pleth} %pred	1.11	1.02–1.21	0.022
MEF ₂₅ %pred	0.92	0.87–0.98	0.013
B. Prediction of mild-marked AHR (PC ₂₀ ≤ 4 mg/mL)			
Variables	OR	95% CI	p-value
BMI	0.76	0.58–1.00	0.050
FeNO	1.03	0.99–1.07	0.138
FRC _{pleth} %pred	1.08	1.01–1.15	0.028
MEF ₂₅ %pred	0.96	0.91–1.00	0.058
C. Prediction of moderate-marked AHR (PC ₂₀ ≤ 1 mg/mL)			
Variables	OR	95% CI	p-value
BMI	0.96	0.77–1.20	0.724
FeNO	1.03	1.00–1.06	0.101
FRC _{pleth} %pred	1.06	1.00–1.13	0.043
MEF ₂₅ %pred	0.99	0.95–1.03	0.571

Significant *p* values are boldtyped.

Our results show that patients presenting with asthma like-symptoms and non-obstructive spirometry

with no evidence for small airways dysfunction and elevated blood eosinophils or FeNO rarely had AHR. Noteworthy, in our cohort only one patient with PC₂₀ ≤ 16 mg/mL did not show increased pretest eosinophil count, FeNO, FRC_{pleth}%pred or decreased pretest MEF₅₀%pred, MEF₂₅%pred, FEF₂₅₋₇₅%pred. It seems that in such patients performing MCT may be omitted or delayed in the diagnostic work-up as they rather need prompt evaluation for other than asthma diseases, which may produce respiratory symptoms. Conversely, we believe that demonstrating impaired small airways function and increased blood eosinophils or FeNO in patients with clinical suspicion of asthma and normal FEV₁/FVC ratio increases the probability of asthma diagnosis. Therefore, especially in highly symptomatic patients and with difficult access to MCT, recording small airway dysfunction may favor immediate introduction of controller medications. In this way, a compromise may be reached to reconcile both physician's need to objectively prove an airway inflammatory process and patient's need to alleviate the symptoms.

In view of the COVID-19 pandemic we would like to emphasize that the MCT is probably associated with generating higher amounts of aerosol compared to baseline spirometry and techniques of airway resistance measurement. We believe that introduction of presented multi-approach baseline assessment integrating measurement of blood eosinophils, FeNO and small airway dysfunction may enable to lower the risk of SARS-CoV-2 transmission in pulmonary function laboratory and give an opportunity to diagnose patients in the setting of restricted access to advanced health services. We are aware that blood eosinophils, FeNO and markers of small airways dysfunction could not serve as ideal surrogates of the presence of AHR. They rather indicate the probability of the presence of certain pathways of AHR, including airway inflammation and small airway obstruction which may be not detectable with the use of classic FEV₁ and (F)VC measurement.

Although the blood eosinophil count is less accurate in the evaluation of airway eosinophilic inflammation than the sputum eosinophil count, we observed significant correlation between blood eosinophilia and the level of AR to methacholine and high diagnostic yield of increased blood eosinophils in discriminating patients with AHR from those with normal AR. Similarly, Schwartz et al., who investigated asymptomatic adults with a history of asthma or wheezing in childhood, family history of asthma or an abnormal spirometry, observed significantly higher blood eosinophil count in subjects with a positive MCT compared to those with a negative MCT (26). Also the correlation between blood eosinophil count and the level of AHR was demonstrated previously (38,39).

Another marker of airway inflammation, FeNO was also previously assessed as a predictor of AHR. Schleich et al. and Pedrosa et al. observed significantly higher values of FeNO among patients with respiratory symptoms and normal FEV₁ value or no bronchial reversibility, who tested positive for AHR (PC₂₀ ≤ 16 mg/mL) compared to those with negative results of MCT (40). In ROC curve analysis they reported that FeNO significantly predicted PC₂₀ ≤ 16 mg/mL with AUC of 0.62 and 0.76 which is in line with our findings (40,41). Schleich et al. propose 34 ppb as a cutoff point of FeNO value for predicting PC₂₀, which offers high specificity (95%) but low sensitivity (35%), whereas Pedrosa calculated optimal cutoff point at 40 ppb with more balanced, comparable with our findings, sensitivity and specificity values (74.3% and 72.5%, respectively) (40). Significant differences in FeNO were also observed by Arıkoğlu et al. between children with asthma who demonstrated mild-marked

AHR and those with PC₂₀ <4 mg/mL (42). They reported that the value of FeNO >28 ppb could effectively discriminate children with asthma with PC₂₀ <4 mg/mL from those with PC₂₀ ≥4 mg/mL (AUC = 0.72) (42). Notably, in above studies higher cutoff values were calculated comparing with our results. On the other hand, Giovannini et al. did not observe significant difference between FeNO in symptomatic patients with normal baseline spirometry with borderline-marked AHR compared to those with normal AR (43).

Similarly to our findings, spirometric measures of small airway function were previously shown to be related to the level of airway responsiveness to methacholine. Raji et. al observed that pretest FEF₂₅₋₇₅% pred. was significantly lower (70.9% ± 19.2% vs 84.2% ± 22.7%) in patients with positive results of MCT comparing to those without AHR. Also Sposato et al. observed significantly lower pretest FEF₂₅₋₇₅%pred in patients with borderline and moderate/severe AHR compared to those with normal airway reactivity assessed by MCT (68.6% ± 19.4% and 74.8% ± 18.8% vs. 82.8% ± 21%, respectively) (8). Furthermore, Currie et al. observed significantly lower baseline FEF₂₅₋₇₅%pred among subjects with mild asthma and borderline AHR compared to those with moderate-marked AHR (71%, vs. 81%). Milota et al., who investigated patients with common variable immunodeficiency, recorded decreased baseline MEF₂₅%pred in patients who tested positive for AHR comparing to those with normal airway responsiveness (76.7% vs. 106.5%) (44).

FRC_{pleth} was previously reported to correlate strongly with distal eosinophilic lung inflammation in asthma patients (25). However, there are only scarce data in the literature regarding the relation between baseline FRC_{pleth} and the level of airway responsiveness to methacholine. Essalhi et al. found that FRC_{pleth} was significantly greater in obese women with asthma comparing to those without asthma diagnosis (45). Furthermore, they demonstrated that increased FRC_{pleth} was independent determinant of airway responsiveness in obese women (45). Also Sood et al. found higher baseline FRC_{pleth} in patients with asthma diagnosed on the basis of positive MCT compared with those with chronic cough and negative MCT and comparing with healthy controls (46). On the other hand, Parker et al. did not record significant difference in FRC_{pleth} between patients referred for MCT because of asthma-like symptoms with confirmed AHR compared to those with normal responsiveness to methacholine (47). However, they noticed significantly higher baseline FRC in patients with ≥20% reduction in FEV₁

during MCT comparing to FEV₁ non-responders in whom methacholine induced $\geq 40\%$ reduction in sG_{aw} (47).

We did not observe significant differences in airway resistance parameters measured using FOT, plethysmography and interrupter technique between patients with different levels of airway responsiveness to methacholine. However, we recorded significant correlation between sRaw and level of AHR, and we recorded that value of sRaw could discriminate subjects with PC₂₀ ≤ 4 mg/mL from those PC₂₀ > 4 mg/mL and subjects with PC₂₀ ≤ 1 mg/mL from those with PC₂₀ > 1 mg/mL. There are limited data in the literature regarding the ability of airway resistance parameters to predict the level of AR to methacholine. Arikoglu et al., similarly to our observations, found that neither R_{FOT_5} nor R_{FOT_5-20} differed significantly between children with asthma with PC₂₀ < 4 mg/mL and ≥ 4 mg/mL (42). However, they recorded significantly higher baseline sR_{aw}%pred. among children with PC₂₀ < 4 mg/mL compared to those with PC₂₀ ≥ 4 mg/mL (42). In line with our findings, Panagou et al. did not record significant relation between baseline R_{int} and the level of AR to methacholine (48).

To establish the value of small airways function measures, blood eosinophils and FeNO in the evaluation of patients suspected of asthma with normal baseline spirometry, the following issues need to be addressed in the future prospective studies on larger group of patients: selection of optimal baseline parameter or set of parameters to predict asthma diagnosis established on the basis of results of AHR, physician opinion and assessment of treatment effects; prognostic value of isolated abnormalities in small airways function and increased blood eosinophils and FeNO for the future development of more pronounced pulmonary function abnormalities.

We acknowledge that our study has several limitations. First of all, the study sample is small, which limits the ability to demonstrate small differences in baseline pulmonary function indices between patients with different level of AR to methacholine. Furthermore, we did not include bronchodilator reversibility in our analysis, because bronchodilator reversibility testing is not performed routinely in our lung function laboratory in patients with no airflow limitation in baseline spirometry. Moreover, as we described previously (30), MCT was performed according to ATS protocol modified by adding the assessment of airway resistance to the routine measurement of FEV₁ at each step of MCT, which extended the time interval between inhalations of successive methacholine solutions (median 9 min, IQR 9–10 min) and

between inhalation of methacholine and the onset of FEV₁ measurement (median 5 min, IQR 4–5 min). We acknowledge that it may have affected the cumulative effect of inhaled methacholine, however intervals remained much lower than reported duration of methacholine action (49). Finally, we present the results of AHR as PC₂₀, which was a routine practice in our lung function laboratory at the moment of data collection, instead of recently recommended provocative dose (PD₂₀) (11).

In conclusion, blood eosinophils, FeNO and markers of small airways dysfunction (MEF₂₅, FEF_{25–75}, FRC_{pleth}) are strongly related to the level of AR to methacholine in patients with asthma-like symptoms and no airflow limitation in baseline spirometry. Borderline-marked AHR could be predicted by increased FRC_{pleth} and decreased MEF₂₅%pred, whereas mild-marked AHR by decreased BMI and increased FRC_{pleth} in this group of patients. In view of limited access to the AHR testing, addition of blood eosinophils, FeNO and markers of small airway dysfunction to the routine baseline lung function evaluation of patients with asthma-like symptoms and non-conclusive spirometry results may facilitate discrimination of patients with high risk of AHR and asthma diagnosis from those with lower risk of AHR who rather need work-up for other diseases. In a time of COVID-19 such approach appears also to be a reasonable compromise between introduction of pulmonary function testing precautions and continuity of respiratory diagnostics.

Declaration of interest

The authors report no conflicts of interest.

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