VOLUME LXXVI, ISSUE 1, JANUARY 2023

ISSN 0043-5147
E-ISSN 2719-342X

Wiadomości Lekarskie
Medical Advances

Official journal of Polish Medical Association has been published since 1928

INDEXED IN PUBMED/MEDLINE, SCOPUS, EMBASE, EBSCO, INDEX COPERNICUS,
POLISH MINISTRY OF EDUCATION AND SCIENCE, POLISH MEDICAL BIBLIOGRAPHY

Wiadomości Lekarskie
Medical Advances

Distribution and Subscriptions:
Bartosz Guterman prenumerata@wydawnictwo-aluna.pl
Graphic design / production:
Grzegorz Sztank www.red-studio.eu

Publisher:
ALUNA Publishing House
ul. Przesmyckiego 29,
05-510 Konstancin – Jeziorna
www.wydawnictwo-aluna.pl
www.wiadomoscilekarskie.pl
www.wiadlek.pl

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ORIGINAL ARTICLE

THE LEVEL OF REACTIVE OXYGEN SPECIES AS A MARKER OF ASTHMA SEVERITY IN CHILDREN

DOI: 10.36740/WLek202301128

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ABSTRACT

The aim of the research was to assess the reactive oxygen species (ROS) levels in granulocytes of patients with asthma.

Materials and methods: The study involved 35 children aged 5 to 17 years. 26 children with persistent asthma, partially controlled course in the period of exacerbation were divided into groups: 1 group - mild asthma (n = 12), group 2 - moderate asthma (n = 7) group 3 - severe asthma (n = 7) and control group included almost healthy children (n = 9).

ROS levels in granulocytes were evaluated using BD FACSDiva™. The spirographic complex was used to assess the function of external respiration

Rresults: The level of ROS in granulocytes of patients with severe asthma was significantly reduced compared with children in the control group and patients with mild and moderate asthma ($p_{1-3} = 0.0003$, $p_{2-3} = 0.0017$, $p\ c-_3 = 0.0150$).

The concentration of ROS in granulocytes ≤ 285 a.u. was prognostically significant with high specificity and sensitivity with severe asthma.

Conclusions: The concentration of ROS levels in neutrophils in patients with severe asthma probably reflected the suppression of their products, which suggests the depletion of the reserve capacity of neutrophils.

Decreased concentrations of reactive oxygen species in children with asthma can be considered as a possible marker of asthma severity.

KEY WORDS: asthma; flow cytometry; granulocytes; reactive oxygen species; children

Wiad Lek. 2023;76(1):205-212

INTRODUCTION

Asthma is a chronic inflammatory disease of the respiratory tract. It is characterized by bronchial hyperreactivity and reversible airflow limitation [1-2]. At present, asthma is one of the most common chronic pathologies among children and adolescents [3].

According to WHO estimates about 300 million people suffer from asthma. Asthma incidence rates are 1-18%. In children, this figure varies 5 to 10% [4 - 5].

There are many diagnostic possibilities for asthma in the world, but achieving control remains an open and relevant topic [6-8].

Identification of biomarkers of pediatric asthma is an active area of research. It can potentially bring great clinical benefits and represent a step forward to individual treatment: the so-called precision medicine [9-12].

It is well known that inflammation of the respiratory tract occupies a major position in the pathogenesis of asthma. Studies suggest that eosinophils and neutrophils are the major cells that contribute to inflammation. Increased neutrophils in sputum, bronchoalveolar lavage fluid (BALF) or biopsy is more common in severe asthma [13 - 16].

Recent studies have found that the bronchial biopsy of children with controlled and uncontrolled severe asthma contains varying amounts of intraepithelial neutrophils. In the group with increased neutrophilia, the number of intraepithelial airway neutrophils correlated with better lung function, better symptom control, and a lower maintenance dose of inhaled glucocorticosteroids [11, 17].

Neutrophils are cells that have a unique function of phagocytosis and form the first line of non-specific protection. An important feature of neutrophils is the "respiratory explosion", which results in the production of reactive oxygen species (ROS). The ability to generate

ROS also characterizes the functional activity of neutrophils, namely the possibility of complete phagocytosis [18 - 20]. ROS plays several important roles in an organism. First, the ROS formation is a natural physiological process that constantly takes place in an organism. Secondly, ROS, formed in increased quantities, act as damage factors. Third, ROS is seen as a signaling system involved in key regulatory mechanisms of the living cell. The resulting ROS are involved in two divergent but ongoing biochemical processes - the catabolism of old and the synthesis of new molecules [21-23].

Various methods are used for in-depth study of asthma biomarkers, but in pediatric practice the technique of conducting some of them is difficult. For example, biopsy and bronchoalveolar lavage are invasive, and the collection of induced sputum has age restrictions [11-12].

Therefore, it is advisable to study the concentration of biomarkers in peripheral blood, as in the most accessible environment in routine practice.

That is why peripheral blood is a promising source of biomarkers.

In this study, we conducted a clinical and prognostic assessment of the levels of reactive oxygen species in granulocytes of children with persistent asthma, considering ROS as a signaling system of functional activity of neutrophils capable of phagocytosis.

THE AIM

The aim of the study: to assess the level of reactive oxygen species (ROS) in granulocytes (neutrophils) in children with asthma.

Tasks of the research: 1) to study the clinical and anamnestic features of asthma in children;

2) assess the ROS levels with varying degrees of persistent asthma;

3) Investigate the relationship between the ROS levels and indicators of the function of external respiration, which reflect the disease severity in childhood asthma at different stages of the disease. 4) Based on the results obtained to create a model for predicting the asthma severity in children.

MATERIALS AND METHODS

DESCRIPTION OF PATIENTS

This was a prospective cohort examination of children from 5 to 17 years with asthma: allergic (Ig E-dependent or Ig E-independent), persistent mild, moderate, severe (2 - 4 degrees of severity), partially controlled, on exacerbation. Patients' diagnoses were in line with accepted GINA 2020 recommendations [4].

Exacerbations were mild to moderate in severity. The study was conducted from September to December 2020 at a children's hospital. The study included all children who were admitted to the pulmonology department and met the criteria for inclusion and exclusion.

Inclusion criteria: patient's age from 5 to 17 years with asthma, patient's with symptoms of asthma exacerbation; in 1-2 days from the beginning of asthma exacerbation; signing informed consent by the both patient's parents and patients older than 14.

Exclusion criteria: children under 5 years of age; patients without written consent to conduct the study; patients with acute bronchitis simple, acute obstructive bronchitis, intermittent asthma, pneumonia; patients diagnosed with remission and controlled asthma; with congenital and chronic cardiopulmonary or neurological diseases; hereditary diseases that lead to changes in the functioning of the respiratory tract, including cystic fibrosis; proven immune deficiency; patients with severe somatic condition and decompensation of vital functions; suspected or confirmed gastroesophageal diseases; patients with neoplasms of any localization; pregnant girls.

Assessment of asthma exacerbation was performed according to the recommendations of GINA 2020 using the criteria of severity of asthma exacerbation [4].

To assess the control used diagnostic tests - c-AST, taking into account the age of children. Test for children aged 4-11 years and 12 years and adolescents [4,21,22]. According to the obtained results and GINA criteria, there was a partially controlled asthma (2 criteria were positive).

Patients received basic treatment for asthma according to the GINA 2020 recommendations [4].

Children with persistent asthma (grade 2 severity), partially controlled during exacerbation, mild exacerbation, received therapy according to step 2 GINA (low-dose ICS and short-acting β2-agonists (if necessary)) [4].

Children with moderate asthma (grade 3), partially controlled during exacerbation, mild exacerbation, received treatment according to GINA step 3 (combination drugs (low-dose ICS / β2-long-acting) and short-acting β2-agonists). for needs)) [4].

Children with persistent severe asthma (grade 4), partially controlled during exacerbation, mild exacerbation, received treatment according to step 4 GINA (combination drugs (medium doses of ICS / β2-long-acting) and short-acting β2-agonists). for needs)) [4].

All patients underwent physical and laboratory examination. The viability and level of reactive oxygen species (ROS) in granulocytes were also studied in children. The study of these indicators was performed in the first two days of hospitalization in the presence of clinical manifestations and wheezing.

The control group consisted of 9 healthy children (of similar age / sex) without any signs of chronic or acute illness during the previous three months who were referred for age control or vaccination. Parents of all patients were informed of the objectives of the study and received written informed consent before enrollment in the study.

Patients were divided into groups depending on the degree of asthma: 1 group - asthma mild persistent (n = 12), 2 group - asthma moderate persistence (n = 7), 3 group - asthma severe persistent (n = 7), the fourth is the control group (n = 9).

Collection of blood samples and preparation of leukocyte suspensions

Blood samples from patients and control subjects who participated in the study were collected in the morning on an empty stomach. Sterile K2EDTA Vacutainers were used for blood collection (IMPROVACUTER Evacuated EDTA K2 Spray Dried PET Tubes, Guangzhou, China). Within two hours after the collection of samples, they were used to obtain leukocyte suspensions. Briefly, 50 µl of blood were lysed using a working solution of BD Pharm LyseTM Lysing Buffer (Becton, Dickinson and Company, BD Bioscience, San Jose, CA, USA).

ROS levels were evaluated in granulocytes using H2DCFDA staining

Leukocyte suspensions were stained with a ROS-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, InvitrogenTM, USA). Its 10 mM stock solution in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was used to prepare a working solution in PBS. Working solution was added to leukocyte suspensions (10 µM). Incubation with H2DCFDA lasted for half an hour in the dark. Simultaneously, 10 µl of 7-aminoactinomycin D (7AAD) was added to the samples. This dye is used to distinguish viable from non-viable cells, since it can penetrate inside the cells only when the cell membrane integrity is compromised. 7AAD becomes fluorescent upon binding to DNA. Thus, non-viable cells are 7AAD-positive (Fig. 1).

DATA ACQUISITION AND POST-ACQUISITION ANALYSIS

The population of granulocytes was allocated using BD FACSDiva™ software (Becton Dickinson, USA) based on forward and side scatters. Thereafter, the region of viable 7AAD-negative granulocytes was gated based on the 7AAD fluorescence acquired in the FL3 channel.

H2DCFDA is known to be converted to a fluorescent dichlorofluorescein (DCF) after the cleavage by esterases inside the cells and subsequent interaction with ROS. DCF fluorescence in viable 7-AAD-negative granulocytes was detected in the FL1 channel by BD FACSCanto™ II Cell Analyzer (BD Biosciences, USA). DCF fluorescence is known to be proportional to intracellular ROS levels. To assess intracellular ROS levels, the mean fluorescence intensity (MFI) of DCF fluorescence in 7AAD-negative granulocytes was compared between groups [26,27].

The assessment of the children's external respiration function was performed using the spirographic complex "SpiroCom AINC.941311.005 I". It was manufactured by the National Aerospace University "HAI" STC of electronic medical devices and technologies "HAI-Medica", Kharkiv, Ukraine (TU U- 33.1-02076 005-2002). The study was performed according to the standard method of spirography.

All statistical calculations were performed using batch program StatSoft STATISTICA version 8 (Tulsa, OK) and MedCalc statistical software versions 17.2.

BIOETHICS

The planned clinical study was carried out after receiving approval by the Ethics and Bioethics Commission of Kharkiv National Medical University on October 2, 2019, protocol № 6 and was conducted in accordance with the principles of the Helsinki Declaration, amended in October 2013.

RESULTS

GENERAL INFORMATION

Of the 28 children surveyed, 42.8% (12) had persistent mild asthma, 25% (7) had persistent moderate asthma, and 25% (7) had persistent severe asthma. No significant statistical difference was found between the groups in the collection of life history and disease. This concerned children's sex and age, the presence of atopy in the patient and close relatives (the presence of atopic dermatitis, allergic rhinitis, allergic diseases in the family, asthma in the family). The laboratory examination revealed no difference in the levels of eosinophils and neutrophils. The increase in IgE level in patients with moderate and severe asthma relative to mild asthma was statistically significant (Table I).

Examination of lung function revealed a statistically significant decrease relative to the severity of asthma: FEV1(Forced Expiratory Volume in one second), FEV1 / FVC (Tiffno test), PEF (Peak Expiratory Flow) were significantly lower in patients with severe asthma relative to mild (Table I).

Correlation between ROS levels in 7AAD-negative granulocytes and FEV1, FEV1 / FVC, PEF.

There were also a direct and positive correlations between ROS levels in 7AAD-negative granulocytes and FEV1 $r = 0.6394$ $p < 0.05$; FEV1 / FVC $r = 0,7322$ $p < 0.05$; PEF $r = 0.6387$ $p < 0.05$.

Table I. Demographic and clinical characteristics of the subjects

Sign	units	Mild asthma (Group 1)	Moderate asthma (Group 2)	Severe asthma (Group 3)	p
	n	n = 12	n = 7	n = 7	
Gender, M/F	n	7/5	1/6	3/4	P1 > 0,05 P2 <0,05. P3 > 0,05
Age, years	Me (Lq; Uq)	9,5 (5,0;17,0)	11,4 (8,0;16,0)	12,7 (7,0;17,0)	p1-2 – 0, 125 p1-3 – 0, 132 p 2-3 – 0, 612
Presence of atopic dermatitis	%, n	58,3% (7/12)	85,7% (6/7)	85,7% (6/7)	p1-2 – 0,1114 p1-3 – 0,5000 p 2-3 – 0,1114
Present of allergic rhinitis	%, n	41,6% (5/12)	57,1% (4/7)	28,5% (2/7)	p1-2 -0,2549 p1-3 -0,2896 p 2-3 -0,1476
Present of allergic disease in relatives	%, n	41,6% (5/12)	71,4% (5/7)	71,4% (5/7)	p1-2 – 0,1193 p1-3 – 0,1193 p 2-3 – 0,5000
Presence of asthma in relatives	%, n	25% (3/12)	42,8% (3/7)	42,8% (3/7)	p1-2 – 0,2135 p1-3 – 0,2135 p 2-3 -0,5000
High eosinophil blood	%, n	8,3% (1/12)	14,2% (1/7)	28,5% (2/7)	p1-2 – 0,6821 p1-3 – 0,2419 p 2-3 – 0,5075
IgE increase, IU/ml	%, n	66,6% (8/12)	100% (7/7)	100% (7/7)	p1-2 – 0,0279 p1-3 – 0,0279 p 2-3 – 1,0000
High neutrophil blood	%, n	0% (0/12)	0% (0/7)	0% (0/7)	p1-2 – 0,5000 p1-3 – 0,5000 p 2-3 – 0,5000
FEV1 %	Me (Lq; Uq)	104,0 (101,0; 122,5) %	100,0 (89,0; 104,0)	78,0 (73,0; 78,0)	KW: H= 16,286; p= 0,0003 MW: p1-2 – 0,0692; p1-3 – 0,0003; p2-3 – 0,0026.
FEV1/FVC %	Me (Lq; Uq)	107,5 (106,5; 110,5) %	102,0 (95,0; 104,0)	92,0,0 (89,0; 97,0)	KW: H= 16,975; p= 0,000 MW: p 1-2 –0,0060; p1-3 – 0,0004; p 2-3 –0,0350.
PEF %	Me (Lq; Uq)	106,0 (104,5; 118,5) %	104,0 (80,0; 109,0)	61,0 (42,0; 69,0)	KW: H= 14,674; p= 0,0007 MW: p1-3 – 0,2907; p1-3 – 0,0004; p 2-3 –0,0040.

KW — Kruskal-Wallis test; Me (Lq; Uq) — median (lower quartile; upper quartile); MW— Mann-Whitney test; p significant with the Bonferroni correction.

Concentration of ROS in neutrophils

In the current study, ROS production by granulocytes was analyzed in patients with different stages of asthma: Me (Lq; Uq) (Group 1) - 393,0 (353,0; 457,0), a.u.; (Group 2) - 355,0 (290,0; 411,0), a.u.; (Group 3) - 274,0 (204,0; 283,0), a.u. H2DCFDA staining indicated that mild and moderate asthma was not associated with reduced ROS levels in 7AAD-negative granulocytes, i.e. viable

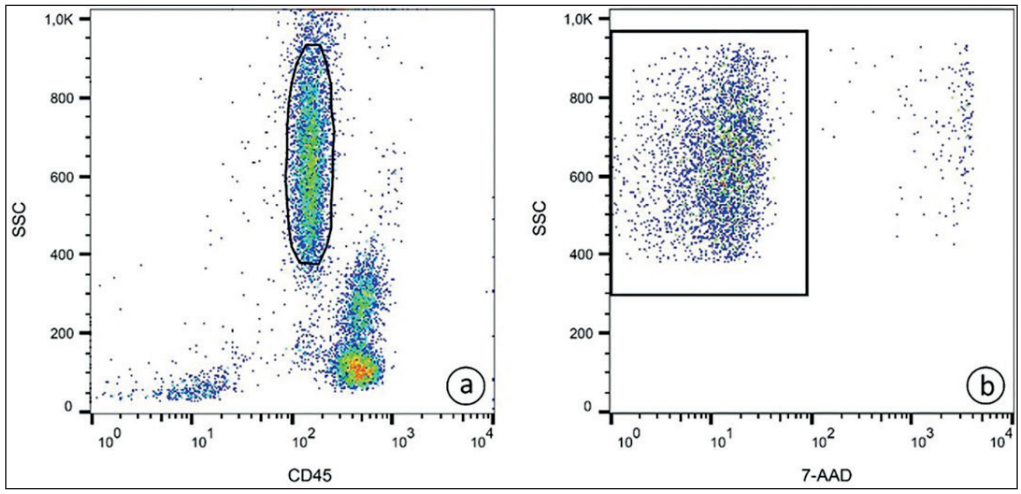

Fig. 1. Representative images that reveal the gating strategy used for isolating the subpopulation of granulocytes in the population of CD45-positive cells (SSC/FL6 dotplot, panel a) and viable cells stained negatively with the DNA intercalator 7-aminoactinomycin D (panel b).

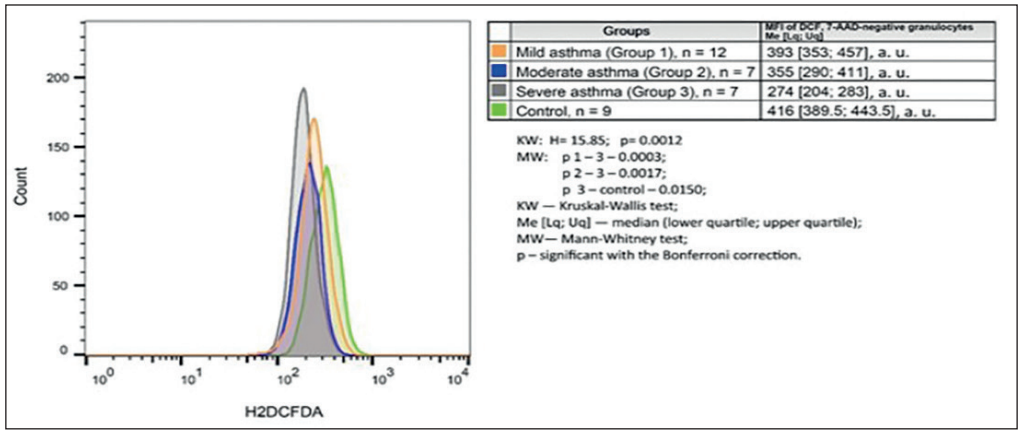

Fig. 2. Representative histograms that demonstrate dichlorofluorescein (DCF) fluorescence in the gated population of viable granulocytes obtained from patients with mild, moderate and severe asthma, as well as healthy individuals. Asthma is associated with a decrease in DCF fluorescence in granulocytes suggesting the reduction of intracellular reactive oxygen species (ROS) levels.

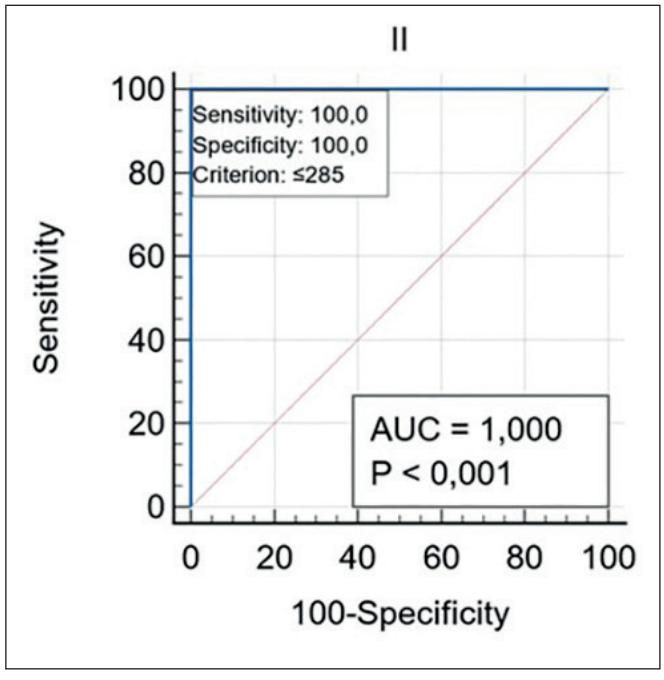

Fig. 3. ROC curves for ROS levels in granulocytes as a biomarker of the severity asthma in children.

cells. No statistically significant differences were found in DCF MFI values. MW: p 1 - 2 = 0.1083; p 1 - c = 0.6996; p 2 - c = 0.0933. However, the severe asthma was accompanied by a statistically significant decrease (p=0,0150) in MFI values of DCF in 7AAD-negative granulocytes. MW p 1 - 3 = 0,0003; p 2 - 3 = 0,0017; p 3 - C = 0,0150. Such changes in MFI values indicate that ROS levels in granulocytes are reduced in patients with severe asthma (Fig. 2).

Prognostic criteria for ROS levels in granulocytes.

ROC analysis was performed to determine the prognostic value of ROS levels in peripheral blood granulocytes. The relationship between the level of ROS in granulocytes and the asthma severity was determined. The limit value for the level of ROS in granulocytes is below 285 a.u. led to a specificity of 100% and a sensitivity of 100% for a predetermined endpoint, namely the formation of severe asthma (Fig. 3).

DISCUSSION

It is known that neutrophils occupy one of the key positions in inflammation of the respiratory tract and are the first line of nonspecific immune defense [13-17]. The neutrophils ability to generate ROS characterizes their functional activity and the possibility of complete phagocytosis [18-20].

Neutrophils play a crucial role in the transmission of redox potential signals due to their early recruitment and the wide variety of ROS released [23].

Researchers have identified the involvement of ROS in many important processes in the body - from damage to recovery. Elevated levels of ROS act as a factor in damage, including asthma [28]. But at the same time, ROS are involved in key regulatory mechanisms of the living cell (catabolism of old and synthesis of new molecules), playing the role of signaling system [21 - 23]. It is noteworthy that ROS generated during the inflammatory process play an important role in the healing and activation of neuroprotective pathways [23]. Scientists associate this with the recovery process.

This study found that patients with varying degrees of asthma had different levels of ROS production in 7AAD-negative granulocytes (neutrophils), ie viable cells in peripheral blood.

In our study, patients with mild to moderate asthma had higher levels of ROS in neutrophils compared with patients with severe asthma, where there was a statistically significant reduction. The results we obtained are no exception. Similar results have occurred in other scientific studies, where the object of study was a severe degree of asthma [11, 17]. In the context of this study, higher neutrophil levels were found to be associated with better asthma control, higher external respiration function, and lower doses of inhaled corticosteroids.

The relationship between ROS and FEV1, FEV1 / FVC, PEF was evaluated in our study. A direct, positive correlation between these indicators was established. This has shown that higher levels of ROS in neutrophils are associated with better lung function and milder asthma.

In a scientific study of cerebral ischemia, early ischemia was associated with an increase in ROS in brain tissue. The researchers concluded that elevated levels of ROS are the result of cerebral ischemia and exacerbation of the disease by inducing cell death, apoptosis and aging due to oxidative stress. In the stage of recovery of brain tissue, ROS play the role of a signaling molecule and may be useful for regulating angiogenesis and preventing tissue damage [22].

Therefore, the higher concentration of ROS in neutrophils in children with mild to moderate asthma can be considered as an assistant in the repair of damaged lung tissue by removing dead cells and cell debris.

The ROC analysis determined the predictive concentrations of ROS in granulocytes (neutrophils) in children with severe asthma. The concentration of ROS in granulocytes ≤ 285 a.u. was prognostically significant with high specificity and sensitivity in children with severe asthma. We did not find similar data in scientific works.

Decreased levels of ROS in granulocytes (neutrophils) in children with varying degrees of asthma can be considered as a possible predictor of its severity.

However, there are limitations. First, there are a small number of patients included in this study. The sample size will be expanded in the future. Second, the study was performed without specifying the heterogeneity of asthma (eosinophilic, neutrophilic or paucigranulocyte phenotype) [29-30].

In addition, one of the limitations of this study was the use of only one biological material for examination - blood. Researchers searching for the role of neutrophils in childhood asthma asthma used bronchoalveolar lavage (BAL), primary bronchial epithelial cells obtained during fibrobronchoscopy, endobronchial cleansing, and biopsy as material [11,17]. But for us, determining the severity markers of childhood asthma in the least invasive way was crucial.

The priority of the current study was to combine clinical characteristics and available biomarkers of bronchial asthma. Further studies of asthma biomarkers in children are needed to improve the prediction of key clinical outcomes and, as a consequence, the development of individualized treatments.

CONCLUSIONS

1. Patients with mild to moderate asthma have higher levels of ROS in neutrophils compared to patients with severe asthma.
2. The ROS concentration in granulocytes of peripheral blood in children with asthma below 285 a.u can be considered as an additional marker of its severity.
3. Higher levels of ROS generated by granulocytes had a positive correlation of external respiration function, which indicated better lung function.

The results of our study will probably be able to influence the further tactics of examination and treatment of patients with asthma.

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We thank all patients and their families for agreeing to participate in our study. He also expresses his sincere gratitude to all the participants who spent their time for their contribution to this study.
This article is part of the research work of the Department of Pediatrics No. 2, with the financial support of Kharkiv National Medical University.

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Conflict of interest:
The Authors declare no conflict of interest.

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Received: 03.06.2022
Accepted: 28.12.2022

A - Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article