ORIGINAL ARTICLES

УДК 616.24-002.5-031.5-092.19
https://doi.org/10.20538/1682-0363-2023-4-92-99



# Notch signaling pathway in the development of imbalanced immune responses in patients with disseminated pulmonary tuberculosis

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## ABSTRACT

**Aim.** To determine the role of the Notch signaling pathway in the regulation of Th1 / Th2 lymphocyte balance in patients with disseminated drug-sensitive (DS) and drug-resistant (DR) pulmonary tuberculosis (PT).

**Materials and methods.** Mononuclear leukocytes were isolated from the venous blood of 13 patients with disseminated PT by density gradient centrifugation. The cells were cultured for 72 h in the complete cell culture medium at 5% $CO_2$ and 37 °C. Preliminarily, CFP10 and ESAT6 mycobacterial antigens or γ-secretase inhibitor DAPT (5 μM / l; 10 μM / l) together with CFP10 and ESAT6 antigens were added to the culture medium. Immunophenotyping of Th1 and Th2 lymphocytes was performed by multicolor flow cytometry by determining the expression of CD4 receptor and intracellular transcription factors T-bet and GATA-3.

**Results.** In patients with disseminated DS and DR PT, an increase in the number of Th1 and Th2 lymphocytes was found in intact cultures. Stimulation of cells with mycobacterial antigens CFP10 and ESAT6 resulted in an increase in the number of $CD4^+T\text{-}bet^+$ and $CD4^+GATA\text{-}3^+$ cells in all comparison groups. Addition of CFP10 and ESAT6 antigens and DAPT (10 μM / l) to the incubation medium was accompanied by a decrease in the number of Th2 lymphocytes in PT patients in both groups. A rise in the number of Th1 cells was registered only in patients with DS PT. Suppression of the Notch signaling pathway with the γ-secretase inhibitor DAPT (10 μM / l) resulted in an increase in the Th1 / Th2 lymphocyte balance in both DS and DR variants of the disease.

**Conclusion.** The Notch signaling pathway has a modulating effect on the differentiation of the key lymphocyte populations that determine the balance between cell-mediated and humoral immune responses to PT. Suppression of the Notch signaling cascade by the γ-secretase inhibitor DAPT (10 μM / l) *in vitro* promotes an increase in the Th1 / Th2 ratio in patients with disseminated DS and DR PT. The positive regulatory effect on the Th1 / Th2 lymphocyte balance allows to consider the Notch signaling pathway as a promising potential target in the development of new approaches to the pathogen-specific therapy for PT.

**Keywords:** Notch signaling pathway, pulmonary tuberculosis, lymphocytes, γ-secretase, helper T cells

**Conflict of interest.** The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

**Source of financing.** The study was performed within the planned research topic of Siberian State Medical University under the program “Molecular medicine” (Molecular and cellular basis of inflammation in socially sensitive pathology).

**Conformity with the principles of ethics.** All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Siberian State Medical University (Protocol No 7924 of 14.10.2019).

**For citation:** Sanina A.E., Serebryakova V.A., Urazova O.I., Gadzhiev A.A. Notch signaling pathway in the development of imbalanced immune responses in patients with disseminated pulmonary tuberculosis. *Bulletin of Siberian Medicine.* 2023;22(4):92–99. https://doi.org/10.20538/1682-0363-2023-4-92-99.

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# Сигнальный путь Notch в развитии дисбаланса иммунных реакций у больных диссеминированным туберкулезом легких

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**РЕЗЮМЕ**

**Цель исследования.** Определить роль сигнального пути Notch в регуляции баланса Th1/Th2-лимфоцитов у больных диссеминированным лекарственно-чувствительным (ЛЧ) и лекарственно-устойчивым (ЛУ) туберкулезом легких (ТЛ).

**Материалы и методы.** Из венозной крови 13 пациентов с диссеминированным ТЛ мононуклеарные лейкоциты выделяли методом градиентного центрифугирования. Клетки культивировали в течение 72 ч в полной питательной среде при 5%-м $CO_2$ и температуре 37 °С, предварительно добавляя в инкубационную среду антигены микобактерий туберкулеза CFP10-ESAT6 или ингибитор γ-секретазы DAPT (5 мкМ/л; 10 мкМ/л) вместе с антигенами CFP10-ESAT6. Иммунофенотипирование Th1- и Th2-лимфоцитов проводили методом проточной лазерной многоцветной цитофлуориметрии посредством определения экспрессии рецептора CD4 и внутриклеточных транскрипционных факторов T-bet и GATA-3.

**Результаты.** У больных диссеминированным ЛЧ и ЛУ ТЛ установлено увеличение количества Th1- и Th2-лимфоцитов в интактных культурах. Стимуляции клеток антигенами микобактерий CFP10-ESAT6 способствовала повышению числа $CD4^+T\text{-}bet^+$ и $CD4^+GATA\text{-}3^+$ клеток во всех группах сравнения. Добавление в инкубационную среду антигенов CFP10-ESAT6 и DAPT (10 мкМ/л) сопровождалось уменьшением количества Th2-лимфоцитов у больных ТЛ обеих групп. Повышение числа Th1-клеток регистрировалось только у пациентов с ЛЧ ТЛ. Подавление сигнального пути Notch с помощью ингибитора γ-секретазы – DAPT (10 мкМ/л) приводило к повышению коэффициента соотношения Th1/Th2-лимфоцитов как при ЛЧ, так и при ЛУ вариантах заболевания.

**Заключение.** Сигнальный путь Notch оказывает модулирующее действие на дифференцировку ключевых популяций лимфоцитов, определяющих динамический баланс клеточно-опосредованных и гуморальных реакций противотуберкулезного иммунитета. Угнетение молекулярного каскада Notch ингибитором γ-секретазы DAPT (10 мкМ/л) в условиях *in vitro* способствует увеличению коэффициента соотношения Th1/Th2 у больных диссеминированным ЛЧ и ЛУ ТЛ. Положительное регулирующее действие на баланс Th1/Th2-клеток позволяет рассматривать сигнальный путь Notch в качестве перспективной потенциальной мишени в разработке новых подходов к патогенетической терапии туберкулеза.

**Ключевые слова:** Notch-сигнальный путь, туберкулез легких, лимфоциты, γ-секретаза, Т-хелперы

**Конфликт интересов.** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

**Источник финансирования.** Работа выполнена в рамках плановой темы НИР Сибирского государственного медицинского университета по программе САЕ «Молекулярная медицина» (Молекулярно-клеточные основы воспаления при социально значимой патологии).

**Соответствие принципам этики.** Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом СибГМУ (протокол № 7924 от 14.10.2019).

**Для цитирования:** Санина А.Е., Серебрякова В.А., Уразова О.И., Гаджиев А.А. Сигнальный путь Notch в развитии дисбаланса иммунных реакций у больных диссеминированным туберкулезом легких. *Бюллетень сибирской медицины.* 2023;22(4):92–99. https://doi.org/10.20538/1682-0363-2023-4-92-99.

## INTRODUCTION

A progressive course of tuberculosis is based on hyperergic inflammation involving a wide range of immunocompetent cells [1; 2]. Th2 lymphocytes, which regulate the development of antibody-mediated immune responses, together with Th1 cells participate in the formation of protective responses [3; 4] and, conversely, can contribute to immune-mediated tissue damage and persistence of mycobacteria [5; 6]. It has been established that a more severe course of pulmonary tuberculosis (PT) is associated with an increase in the IL-4 mRNA concentration in the blood [6; 7], high titers of antigen-specific IgG, and increased expression of suppressor of cytokine signaling SOCS3 mRNA [7].

Multiple interactions between cells of the immune system are determined not only by cytokines and chemokines (IFNγ, TNFα, IL-4, IL-12, IL-27, etc.), but also by receptor – ligand interactions [8; 9]. Notch ligands and receptors associated with the intracellular signaling cascade regulating cell differentiation occupy a prominent place among receptor cooperation mechanisms [10]. Dysfunction of molecular mechanisms in the Notch signaling pathway at any stage of implementation (expression of receptors, ligands, enzyme activity, etc.) can contribute to disruption of an effective immune response against Mycobacterium tuberculosis and progression of the disease. Thus, in experiments on murine macrophages, it was established that a BCG-induced increase in the expression of Notch1 receptor protein and subsequent activation of its signaling pathway led to a rise in the expression of SOCS3 protein that provides negative regulation of cytokine signaling [11]. The demonstrated increase in leukocyte expression of molecules initiating the Notch signaling cascade (Notch1/2 and DLL4 mRNA) in patients with PT without changes in the expression of their target genes (Hes1, Hey1) [12] leaves the question about the role of the Notch signaling pathway in the immunopathogenesis of PT open.

One of the molecular approaches to evaluate the role of the Notch signaling cascade in the pathogenesis of various immune-mediated diseases is inhibition of the activity of γ-secretase, a key proteolytic enzyme that initiates the release of the Notch intracellular domain [13]. The properties of the γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-1-alanyl]-s-phenylglycine t-butyl ester) are being actively studied as a potential drug for treatment of cancer, as well as neurological, cardiovascular, and cerebrovascular diseases [14].

The aim of the study was to determine the role of the Notch signaling pathway in the regulation of Th1 / Th2 lymphocyte balance in patients with disseminated drug-sensitive (DS) and drug-resistant (DR) PT.

## MATERIALS AND METHODS

The study involved 13 patients with newly diagnosed disseminated PT (mean age 47.3 ± 5.21 years), who were hospitalized in Tomsk Phthisiopulmonology Medical Center.

The patients were divided into two groups depending on the sensitivity of Mycobacterium tuberculosis to anti-TB drugs: group 1 encompassed 7 patients with bacteriologically confirmed Mycobacterium tuberculosis resistant to one or more anti-TB drugs (isoniazid and rifampicin); group 2 included 6 patients excreting drug-sensitive Mycobacterium tuberculosis. The control group consisted of 10 healthy volunteers with comparable age and gender characteristics.

The study material was whole venous blood taken before treatment with anti-TB drugs. Mononuclear leukocytes were isolated from the blood by density gradient centrifugation (ρ = 1.077 g / ml). Mycobacterium tuberculosis antigens (AG) CFP10 and ESAT6 (Diaskintest, Generium, Russia) at a dose of 10 μg / ml or the γ-secretase inhibitor (DAPT, Tocris Bioscience, UK) at a dose of 5 or 10 μM / l, pre-dissolved in 0.1% dimethyl sulfoxide (DMSO) solution (Sigma-Aldrich, USA), together with CFP10 and ESAT6 were added to the incubation medium. The indicated dose was determined by evaluating the cytotoxicity of the tuberculosis recombinant allergen containing CFP10/ESAT-6 protein by the MTT assay. DMSO and the γ-secretase inhibitor at the indicated concentrations did not cause cell death *in vitro*. Cells were cultured in the RPMI-1640 medium with L-glutamine (Biolot LLC, Russia). The cells were incubated in 5% $CO_2$ at 37 °C for 72 h. Immunophenotyping of Th1 and Th2 lymphocytes was performed by multicolor flow cytometry by determining the expression of surface CD4 receptor (FITC, BD Biosciences, USA) and intracellular transcription factors – T-bet (Alexa Fluor 405, R&D Systems Inc., USA) and GATA-3 (PerCP-eFluor 710, BD Biosciences, USA).

The statistical data were processed using IBM SPSS statistics 25 (Statistical Package for the Social Sciences, USA) and Microsoft Office 2013. The Shapiro – Wilk test was used to check the data for normality of distribution. The data were presented as the median (*Me*) and the 25th and 75th percentiles ($Q_1$ and $Q_3$), as quantitative variables in the study groups

did not follow normal distribution. The nonparametric Mann – Whitney *U* test was used to estimate the level of significance of differences in quantitative variables between the study samples. The Wilcoxon test was used to assess the significance of differences in dependent data within the group. The results of the statistical analysis were considered significant at $p < 0.05$.

## RESULTS

The study showed that the development of disseminated PT was accompanied by an increase in the number of $CD4^+T\text{-}bet^+$ cells. Thus, in patients with DS PT, the number of Th1 lymphocytes exceeded similar parameters in healthy donors by 2 times ($p < 0.01$) and in patients with DR PT –by 1.7 times ($p < 0.01$). The Th2 lymphocyte count in intact cultures in PT patients with different types of mycobacterial resistance was 2.5 times higher ($p < 0.001$) than in healthy individuals (Table 1).

After stimulation of the cells with mycobacterial antigens CFP10 and ESAT6, an increase in the number of Th1 and Th2 cells was registered in all groups under study.

Table 1

**Relative content of Th1 and Th2 lymphocytes in peripheral blood (% of the total lymphocyte count) in patients with disseminated pulmonary tuberculosis, *Me* ($Q_1$–$Q_3$)**

| Parameters | Healthy donors | Patients with pulmonary tuberculosis | |
|---|---|---|---|
| | | Drug-sensitive | Drug-resistant |
| Th1 lymphocytes ($CD4^+T\text{-}bet^+$) | | | |
| Intact culture | 1.25 (1.12–1.37) | 2.51 (2.48–2.59)<br>$p_1 < 0.001$ | 2.19 (2.17–2.22)<br>$p_1 < 0.001$<br>$p_4 = 0.008$ |
| With added AG (CFP10 and ESAT6) | 1.30 (1.18–1.42)<br>$p_2 = 0.012$ | 2.54 (2.51–2.66)<br>$p_1 < 0.001$<br>$p_2 = 0.041$ | 2.34 (2.31–2.34)<br>$p_1 < 0.001$<br>$p_2 = 0.043$<br>$p_4 = 0.008$ |
| With added AG and DAPT (5 μM / l) | 1.37 (1.21–1.44)<br>$p_2 = 0.012$<br>$p_3 = 0.012$ | 2.56 (2.52–2.64)<br>$p_1 < 0.001$ | 2.30 (2.27–2.31)<br>$p_1 < 0.001$<br>$p_2 = 0.042$<br>$p_4 = 0.008$ |
| With added AG and DAPT (10 μM / l) | 1.95 (1.7–2.04)<br>$p_2 = 0.012$<br>$p_3 = 0.012$ | 2.68 (2.63–2.73)<br>$p_1 < 0.001$<br>$p_2 = 0.043$<br>$p_3 = 0.043$ | 2.34 (2.29–2.37)<br>$p_1 < 0.001$<br>$p_4 = 0.008$ |
| Th2 lymphocytes ($CD4^+GATA\text{-}3^+$) | | | |
| Intact culture | 1.04 (0.99–1.01) | 2.57 (2.49–2.63)<br>$p_1 < 0.001$ | 2.67 (2.65–2.69)<br>$p_1 < 0.001$ |
| With added AG (CFP10 and ESAT6) | 1.12 (1.08–1.14)<br>$p_2 = 0.012$ | 2.73 (2.64–2.73)<br>$p_1 < 0.001$<br>$p_2 = 0.043$ | 2.70 (2.68–2.71)<br>$p_1 < 0.001$<br>$p_2 = 0.043$ |
| With added AG and DAPT (5 μM / l) | 0.91 (0.82–0.98)<br>$p_3 = 0.012$ | 2.65 (2.61–2.65)<br>$p_1 < 0.001$ | 2.68 (2.62–2.69)<br>$p_1 < 0.001$ |
| With added AG and DAPT (10 μM / l) | 0.68 (0.63–0.72)<br>$p_2 = 0.012$<br>$p_3 = 0.012$ | 2.19 (2.18–2.21)<br>$p_1 < 0.001$<br>$p_2 = 0.043$<br>$p_3 = 0.043$ | 2.18 (2.13–2.19)<br>$p_1 < 0.001$<br>$p_2 = 0.043$<br>$p_3 = 0.043$ |

Note (here and Table 2): $p_1$ – the level of statistical significance of differences compared to similar parameters in healthy donors; $p_2$ – in the intact culture; $p_3$ – during antigen stimulation (AG); $p_4$ – in patients with DS PT; DAPT – N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-s-phenylglycine t-butyl ester.

The analysis of the population composition of lymphocytes in patients with DS and DR PT after sequential addition of the γ-secretase inhibitor (DAPT) at a concentration of 5 μM / l to the intact cultures in combination with CFP10 and ESAT6 antigens did not reveal any significant differences from the corresponding data obtained when stimulating the cells with antigens alone. A significant ($p = 0.012$)

decrease in $CD4^+GATA\text{-}3^+$ cells and an increase in $CD4^+T\text{-}bet^+$ lymphocytes was registered in healthy donors (Table 1).

Increasing the DAPT concentration to 10 μM / l led to a decrease in the proportion of Th2 cells in the cell cultures in PT patients in both groups ($p = 0.043$) and healthy donors ($p = 0.012$). Changes in the number of $CD4^+T\text{-}bet^+$ lymphocytes were not unequivocal. Thus, an increase in the number of Th1 cells was registered only in patients with DS PT ($p = 0.043$) and in the control group ($p = 0.012$). The number of $CD4^+T\text{-}bet^+$ lymphocytes in DR PT patients was comparable with the same indices obtained during incubation of the cells with CFP10 and ESAT6 antigens.

The comparison of the results obtained between the groups of PT patients showed that under all applied cell culture conditions, the number of Th1 lymphocytes in patients with DR PT was smaller ($p = 0.008$) than in patients with DS PT.

The calculation of the Th1 / Th2 lymphocyte ratio revealed significant differences for the indices obtained during incubation of mononuclear leukocytes with CFP10 and ESAT6 and DAPT (10 μM / l). Inhibition of the Notch signaling pathway resulted in the increased Th1 / Th2 cell ratio relative to the intact and CFP10 – ESAT6-stimulated cultures in PT patients ($p_3 = 0.043$) and healthy donors ($p_3 = 0.012$). There were no significant differences between the groups of patients with different mycobacterial sensitivity (Table 2).

Table 2

**Th1 / Th2 cell ratio in patients with disseminated pulmonary tuberculosis, %, *Me* ($Q_1$–$Q_3$)**

| Th1/Th2 ratio | Healthy donors | Patients with pulmonary tuberculosis | |
|---|---|---|---|
| | | Drug-sensitive | Drug-resistant |
| Baseline | 1.2 (1.13–1.25) | 0.96 (0.94–0.97) | 0.93 (0.88–0.95) |
| With added AG (CFP10 and ESAT6) | 1.16 (1.09–1.24) | 0.96 (0.95–0.99) | 0.95 (0.94–0.95) |
| With added AG and DAPT (10 μM / l) | 2.88 (2.68–2.82)<br>$p_2 = 0.012$<br>$p_3 = 0.012$ | 1.27 (1.21–1.28)<br>$p_1 = 0.003$<br>$p_2 = 0.043$<br>$p_3 = 0.043$ | 1.25 (1.24–1.25)<br>$p_1 = 0.003$<br>$p_2 = 0.043$<br>$p_3 = 0.043$ |

## DISCUSSION

Protective control over the infectious process caused by Mycobacterium tuberculosis is ensured by cooperative interaction of a variety of immunocompetent cells, realized via juxtacrine and paracrine signaling mechanisms. The role of the main population ensuring the development of the adaptive immune response belongs to the pool of antigen-specific $CD4^+$ T lymphocytes [15]. Their crucial role in the pathogenesis of PT is determined by their ability to increase phagocytic activity of macrophages [16], induce chemokine-mediated migration of $CD8^+$ T cells, their cytolytic activity, and their secretion of cytokines (IFNγ, TNFα) and granzymes [15], and is also confirmed by a high risk of disease development in HIV patients [15; 17; 18].

The increased proportion of Th1 and Th2 lymphocytes in the intact cultures of patients with PT indicates complex involvement of cell-mediated and humoral immune responses in the development of a protective response against Mycobacterium tuberculosis. A disseminated course of PT indicates the failure of the responses, aimed at restraining primary mycobacterial infection. An increase in the number of Treg and Th2 cells can be considered as one of the factors contributing to the progression of the pathological process.

Highly specific molecules ESAT6 and CFP10, secreted only by dividing Mycobacterium tuberculosis, play a key role in the development of tuberculosis infection [19]. The ESAT-6 protein has lytic activity, promotes pathogen entry into the cell, and destabilizes phagosomes, allowing mycobacteria to enter the macrophage cytosol and avoid lysis. The CFP-10 antigen forms a complex with ESAT-6 and ensures its delivery to the site of action [20]. The single recombinant ESAT-6 –CFP10 protein is designed to evaluate the cell-mediated immune response to Mycobacterium tuberculosis. The increased number of Th1 and Th2 lymphocytes recorded in all the studied samples in response to stimulation of the cell cultures with mycobacterial antigens CFP10 and ESAT6 reflects the physiological cellular response and may indicate a preserved antigen recognition function and relatively effective intercellular cooperation.

The question on the functional significance of Th2 lymphocytes in the pathogenesis of the progressive course of tuberculosis remains open. Classical antibody-mediated immune responses (opsonization,

complement activation, phagocytosis, lysosomal degradation) can potentially be effective against Mycobacterium tuberculosis [3]. The leading cytokine of humoral immune response is IL-4, produced by Th2 lymphocytes. Molecular mechanisms of IL-4 involvement in immune responses are associated with suppression of TNFα-induced apoptosis in infected cells, reduction of iNOS activity, and enhancement of proliferation of antigen-specific regulatory T lymphocytes [5]. The works by domestic [21; 22] and foreign authors [23–25] established that the polarization of the immune response toward Treg- and Th2-dependent responses is a key element of the immune imbalance in tuberculosis infection. The intensity of the functional activity of Th2 lymphocytes, as well as the titer and spectrum of antibodies formed by plasma cells may be crucial. In view of this, the established decrease in the proportion of Th2 lymphocytes in the cell cultures of PT patients in both groups under the effect of DAPT (10 μM / l) with a parallel increase in the number of Th1 cells in DS PT can be considered as a possible mechanism contributing to the restoration of an effective dynamic balance between the main populations regulating the intensity of destructive processes and providing protective control over the spread of the infection.

A recorded and maintained under various experimental conditions smaller (than in DS PT) number of Th1 lymphocytes in patients with DR PT proves significant disruptions of intercellular cooperation mechanisms, induced by resistant strains of mycobacteria among others. It was established that the development of DR PT is associated with a significant increase in the number and activity of immunosuppressive regulatory T cells (Treg), their production of IL-10, deficiency of NK cells, IFNγ, and IL-2 (the main factor ensuring proliferation of antigen-specific lymphocytes) [24; 26]. Therefore, a possible reason for the initially smaller number of Th1 lymphocytes in DS PT patients in the intact culture and the preservation of this trend when the cells were stimulated with antigens could be the mechanisms induced by drug-resistant mycobacteria to disrupt cell-mediated immune responses that contribute to the persistence of the pathogen and progression of the disease.

The Th1 / Th2 ratio to a certain extent reflects the immune pattern of T lymphocytes [4; 27]. With respect to mycobacterial infection, the predominance of Th1 cells and cell-mediated immune responses provides effective protection against the pathogen, while the predominance of Th2 lymphocytes and humoral immune responses facilitates the development of hyperergic inflammation with immune-mediated tissue damage. The increase in the Th1 / Th2 ratio under the effect of DAPT (10 μM / l) in PT patients compared to the intact and antigen-stimulated cultures (as well as their compliance with baseline values in healthy donors) can be considered as a possible mechanism regulating the restoration of a balance between lymphocyte populations through inhibition of the Notch signaling pathway, which may contribute to more effective development of the immune response and slow down destructive processes.

## CONCLUSION

The Notch signaling pathway has a modulating effect on the differentiation of key lymphocyte populations that determine the dynamic balance between cell-mediated and humoral immune responses. Inhibition of the Notch signaling cascade by the γ-secretase inhibitor DAPT (at a dose of 10 μM / l) under *in vitro* conditions promotes an increase in the Th1 / Th2 ratio in patients with disseminated DS and DR PT. The positive regulatory effect on the Th1 / Th2 balance allows the Notch signaling pathway to be considered as a promising potential target in pathogen-specific therapy for PT. The contribution of the Notch signaling pathway to the regulation of differentiation of other T lymphocyte populations (Th17, Treg) involved in the immunopathogenesis of PT requires further research.

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Received 11.04.2023;
approved after peer review 18.04.2023;
accepted 25.05.2023