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# Association of Toll-like receptor polymorphism and gene expression level with the risk of developing chronic obstructive pulmonary disease (COPD) and its severity

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## ABSTRACT

**Aim.** To determine how genetic factors of innate immunity influence the risk of development and features of the course of COPD.

**Materials and methods.** The study included 103 patients diagnosed with chronic obstructive pulmonary disease and 47 apparently healthy people without any chronic bronchopulmonary pathologies. The expression level of TLR genes and alleles of rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs3806790 (*TLR4*), rs5743810 (*TLR6*), rs3804880 (*TLR8*) single nucleotide polymorphisms were analyzed via real-time polymerase chain reaction (PCR).

**Results.** Several trends were observed: an increase in the proportion of GG homozygotes in the rs5743810 (*TLR6*) locus in patients with severe COPD and a negative correlation between *TLR2* and *TLR6* gene expression level and oxygen saturation in blood, dyspnea and COPD severity.

**Conclusion.** No statistically significant association with rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) single nucleotide polymorphisms was found. The observed trend toward an increase in TLR gene expression may be associated with the remodeling of lung tissues and activation of the immune response that occur during COPD.

**Keywords**: COPD, TLR, single nucleotide polymorphisms, gene expression

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

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**Conformity with the principles of ethics.** The study complies with the ethical principles developed in accordance with the *Ethical Principles for Medical Research Involving Human Participants* in WMA Declaration of Helsinki with amendments of 2000 and the *Rules of Good Clinical Practice of the Russian Federation* approved by Order No. 266 of the Ministry of Health of the Russian Federation dated 19 June 2003. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee of the Medical Institute of the Peoples' Friendship University of Russia (Protocol No. 30 of 17.06.2021).

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# Ассоциация полиморфизма и уровня экспрессии генов Toll-подобных рецепторов с риском развития и тяжестью течения хронической обструктивной болезни легких

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**РЕЗЮМЕ**

**Цель:** определение вклада генетических факторов врожденного иммунитета в риск развития и особенности течения хронической обструктивной болезни легких (ХОБЛ).

**Материалы и методы.** В исследование включены 103 пациента с диагнозом «хроническая обструктивная болезнь легких» и 47 условно здоровых человек без хронической бронхолегочной патологии. Определен уровень экспрессии генов *TLR* и аллели однонуклеотидных полиморфизмов rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs3806790 (*TLR4*), rs5743810 (*TLR6*), rs3804880 (*TLR8*) методом полимеразной цепной реакции в режиме реального времени.

**Результаты.** Выявлена тенденция к увеличению доли гомозигот GG в локусе rs5743810 (*TLR6*) у пациентов с тяжелым течением ХОБЛ и обратная корреляция уровня экспрессии генов *TLR2* и *TLR6* с сатурацией кислорода в крови, выраженностью одышки и тяжестью течения заболевания.

**Заключение.** Для однонуклеотидных полиморфизмов rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) не обнаружено статистически значимой ассоциации. Наблюдаемые тенденции повышения уровня экспрессии генов *TLR* могут быть связаны с возникающим в процессе течения ХОБЛ ремоделированием легочных тканей и активацией пути иммунного ответа.

**Ключевые слова:** ХОБЛ, TLR, однонуклеотидные полиморфизмы, экспрессия генов

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

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## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a heterogeneous lung disease characterized by progressive airflow limitation due to the development of obliterative bronchiolitis, chronic bronchitis, emphysema, and lung tissue remodeling in response to inhaled particles and gasses [1]. These processes have a progressive course associated with the development of inflammation.

COPD exacerbations lead to a progressive decrease in lung capacity, increasing respiratory insufficiency, disease progression and are one of the most frequent reasons for patients to seek emergency medical care, which is associated with significant economic costs [1].

Smoking is a major risk factor for COPD, although not all smokers develop clinically significant lung tissue damage, which indicates that apart from pollutants there are also other factors (including genetics) that influence the development of COPD. According to the literature, Toll-like receptors (TLRs) mediate many cellular immunity responses, including the cytokine response [2]. Activation of these receptors occurs due to the binding of pathogen molecular structures (pathogen-associated molecular patterns, PAMPs) and tissue damage products (damage-associated molecular patterns, DAMPs). Innate immunity activation caused by chronic inflammatory respiratory diseases could be associated with the immune system gene polymorphisms. Specifically, with single nucleotide polymorphisms (SNPs) in TLR genes.

TLRs are mediators of smoking-induced inflammation. Smokers have an increased *TLR2* expression in monocyte cells [3]. Increased expression levels of *TLR4* and *TLR9* are associated with inflammatory processes of the lower airway tissues in patients with COPD [4]. Cigarette smoke-induced oxidative stress and DAMP-induced inflammation are important mechanisms of the immune response[5].

Organic dust-stimulated IL-6 production may be associated with one or more synonymous SNP variants in the *TLR1* gene [6]. rs1898830 (*TLR2*) and rs11938228 (*TLR2*) are associated with accelerated FEV-1 decline and higher inflammatory cell counts in sputum [7]. A population-based study reported an association between rs4986790 (*TLR4*), rs4986791 (*TLR4*), and rs5743708 (*TLR2*) polymorphisms and the development of COPD [8]. A slight association was found between the rs5743810 (*TLR6*) polymorphism and the risk of developing chronic lower respiratory tract diseases [9]. Further studies are needed to clarify the role of TLR gene polymorphisms in COPD.

Thus, the aim of this study was to determine how genetic factors of innate immunity influence the risk of development and features of the course of COPD..

## MATERIALS AND METHODS

A case-control study was conducted using biological material obtained during the period from January 2022 to February 2024 at the V.V. Vinogradov City Clinical Hospital of the Moscow Health Department. The study complies with the ethical principles developed in accordance with the *Ethical Principles for Medical Research Involving Human Participants* in WMA Declaration of Helsinki (with amendments of 2000) and the *Rules of Good Clinical Practice of the Russian Federation* approved by Order No. 266 of the Ministry of Health of the Russian Federation dated 19 June 2003.

The COPD group included patients aged 40 to 70 with a clinically confirmed diagnosis established by a pulmonologist 12 or more months prior to inclusion in the study, smokers at the time of inclusion in the study (smoking index of more than 10 pack-years), hospitalized patients with a COPD exacerbation. All of the participants signed an informed consent.

The study did not include those patients with COPD who also suffered from severe concomitant pathologies, oncological or mental diseases, patients taking medications that may cause lung tissue damage as a side effect, patients who were vaccinated against pneumococcus, as well as patients with other chronic diseases of the bronchopulmonary system, including bronchial asthma.

Exclusion criteria were clinical, laboratory or instrumental signs discovered during medical examination that indicated the absence of COPD or were indicative of other causes for lung tissue damage.

All patients with COPD underwent physical examination and participated in spirometry with a bronchodilator reversibility test (in accordance with ATS requirements) [10]. The severity of COPD was assessed using the BODE index (B – body mass index, O – obstruction, D – dyspnea, E – exercise tolerance) and the scale proposed by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [1]. To exclude other bronchopulmonary pathologies and to clarify the nature of changes in the lung tissue, multispiral computed tomography of the chest organs (MSCT CO) was performed.

The control group included patients with a smoking history of at least 10 pack-years with $SatO_2 \geq 95\%$ saturation who showed no evidence of any respiratory diseases during spirometry with a bronchodilator test and MSCT CO, as well as showed no signs of the right ventricular dysfunction or elevation of mean pulmonary arterial pressure during echocardiography (ECHO CG).

Whole blood samples were collected anonymously in vacutainers with $K_3$EDTA as anticoagulant for genetic studies. Biological material was stored for no more than 4 hours before the nucleic acid mixture was isolated. The study used reagents and kits manufactured at the Central Research Institute of Epidemiology (AmpliSens, Moscow, Russia). SNP alleles and TLR gene expression levels were determined via real-time polymerase chain reaction (PCR). Allele detection of rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) polymorphisms was performed in accordance with the previously described methodology [11].

Reverse transcription reaction was performed using the REVERTA-L reagent kit. Method of normalization of genes with relatively stable expression (housekeeping genes) – *HPRT1*, *SDHA*, *GAPDH* and *TBP* – was used

to calculate the expression level of target genes (*TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR8*). Normalization index was formed using the *BestKeeper* algorithm [12] in order to select genes with the smallest spread of cycle threshold (Ct) values. Information about the development and optimization of the method for determining expression level was described in earlier studies [13].

Statistical analysis was performed using the R environment (version 4.4.0), including standard contingency table analysis functions: Pearson's $\chi 2$ test and Fisher's exact test. Association analysis and odds ratio (OR) estimation were performed using SNPassoc [14] and epitools [15] packages. The Holm – Bonferroni method was used to correct multiple comparisons. Results were considered statistically significant at $p < 0.05$. All graphical results were obtained using ggplot2 [16] and ggstatsplot [17] packages.

## RESULTS

The COPD group ($n$ = 103) included patients with a diagnosis confirmed by a pulmonologist. There was an irregular distribution among the study participants by sex (80 men and 23 women), the mean age of the participants was 67.5 ± 11.6 years, and the mean smoking history was 71 (43; 88) pack-days. In the control group of apparently healthy smokers ($n$ = 47), the mean age was 61.6 ± 9.4 years and the mean smoking history was 65 (32; 79) pack-days. A comparison of clinical and demographic characteristics revealed that the groups differed significantly in age ($p$ = 0.0025). Clinical and demographic characteristics of the study groups are presented in Table 1.

Table 1

**Characteristics of the studied groups**

| Parameter | COPD ($n$ = 103) | Control ($n$ = 47) |
|---|---|---|
| Sex, $n$ (%) | | |
| male, 117 (72.2%) | 80 (77.7%) | 36 (76.6%) |
| female, 45 (27.8%) | 23 (22.3%) | 11 (23.4%) |
| Age, years | 69 (60.5–74) | 61 (54–68) |
| Dyspnea, mMRC score | 3 (2–3) | – |
| Smoking index, pack-years | 71 (43; 88) | 65 (32; 79) |
| FEV1, % of predicted (after bronchodilator test) | 47 (45; 59) | 92 (86; 98) |
| FVC, % of predicted (after bronchodilator test) | 73 (68; 82) | 88 (81; 97) |
| FEV1/ FVC (after bronchodilator test) | 0.52 (0.41; 0.64) | 0.83 (0,76; 0.91) |
| Saturation | 93 (90–95) | 97 (95; 98) |
| Obstruction severity on the GOLD scale | 3 (2–3) | 0 |
| Severity on the BODE scale | 2 (1–3) | 1 (0; 1) |

Note. FEV1 – forced expiratory volume in 1 second; FVC – forced vital capacity.

rs5743551 (*TLR1*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), and rs3764880 (*TLR8*) polymorphic loci were analyzed in order to determine whether there was an association between them and the risk of development and severity of COPD. The studied groups were not significantly different in the frequency of alleles of the studied SNPs (Table 2). rs5743708 (*TLR2*) locus was the exception. For this locus, frequency of a rare A allele was significantly lower (0.5%) in the COPD group than in the control group (9%). Thus, a rare allele may be associated with a protective effect — the risk of COPD in its carriers is lower than in carriers of a wild-type allele (odds ratio (OR) = 0.05; 95% confidence interval (CI) = 0.01–0.43; $p$ = 0.0005).

The COPD group was stratified into four subgroups by disease severity according to the spirometric classification of the severity of bronchial obstruction and recommendations [1]. An analysis of the allele frequency distribution in the studied subgroups was carried out. There were no statistically significant differences between the groups in allele frequency for the studied polymorphic loci. For the rs5743810 (*TLR6*) polymorphism, the number of GG homozygotes tended to increase from the group with moderate disease severity to the group with severe COPD, while the number of rare AA homozygotes decreased and the frequency of heterozygotes remained the same. Table 3 shows the distribution of genotypes in the rs5743810 (*TLR6*) locus and the statistical significance of differences between groups 1 and 3.

During the next stage of the study, the expression levels of TLR genes in the control group ($n$ = 47) and the COPD group ($n$ = 33) were analyzed. Based on the stability analysis of housekeeping genes in the analyzed samples, a complex *BestKeeper* index was formed. The index was based on the geometric mean Ct value of the two most stable genes — *HPRT1* and *SDHA*. Normalization allows, to a certain extent, to exclude the influence of the quality and quantity differences of the source material on the obtained values. In order to determine the expression level differences between patients with different disease severity, they were divided into subgroups for each analyzed parameter. In the group of patients with a saturation level ($SatO_2$) below normal (<95%), there was an increased expression of the *TLR2* and *TLR6* genes. The results are shown in Figure 1. There was a similar trend for the other *TLR* genes but the comparison results were not statistically significant ($p > 0.05$).

Analysis of the TLR gene expression level between subgroups of patients using the modified Medical Research Council Dyspnea Scale (mMRC) [18] showed an increase in the expression of *TLR2* and *TLR6* genes with increasing severity of dyspnea. The results of the analysis are shown in Figure 2.

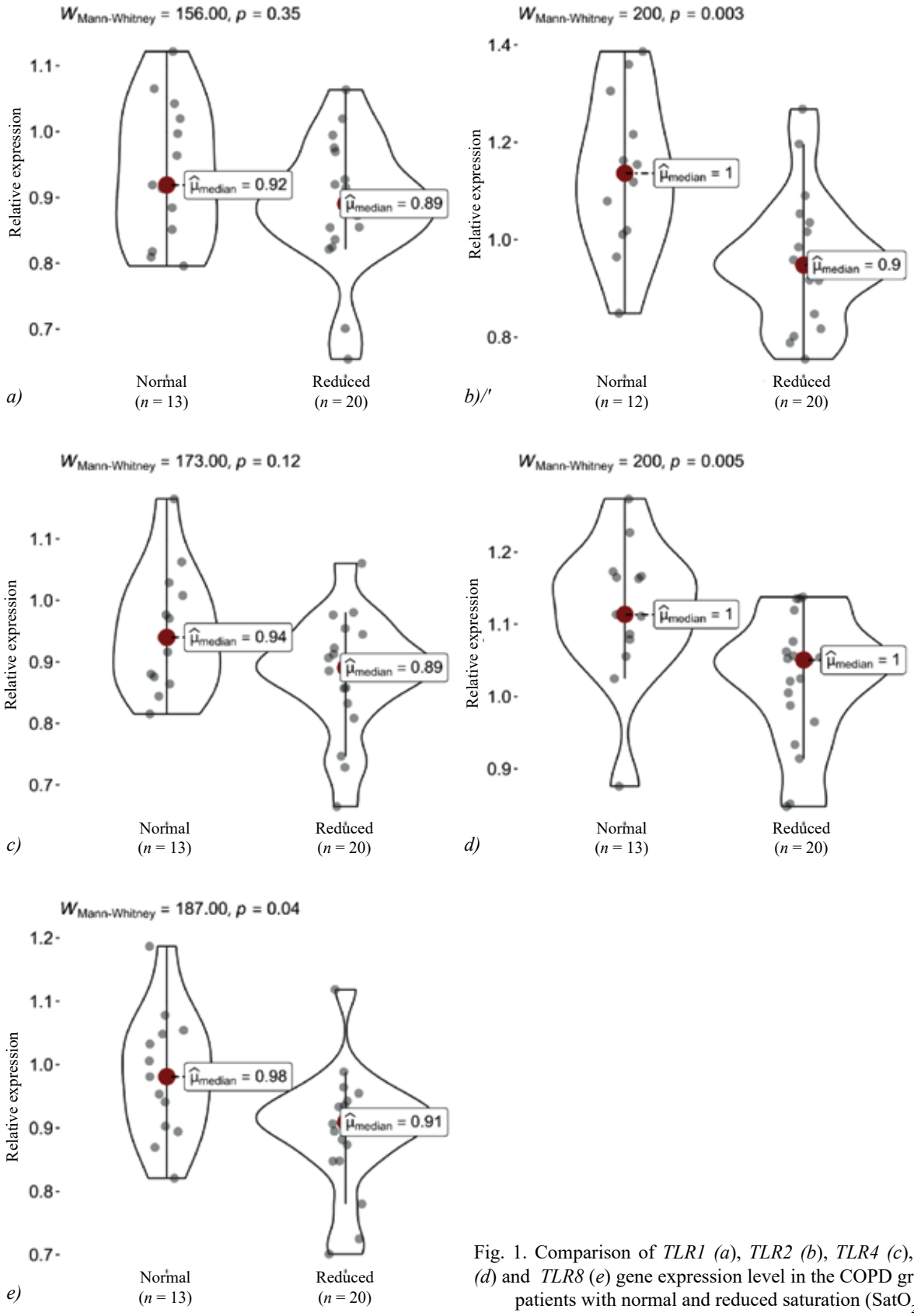


Fig. 1. Comparison of *TLR1 (a)*, *TLR2 (b)*, *TLR4 (c)*, *TLR6 (d)* and *TLR8* (*e*) gene expression level in the COPD group of patients with normal and reduced saturation ($SatO_2$)

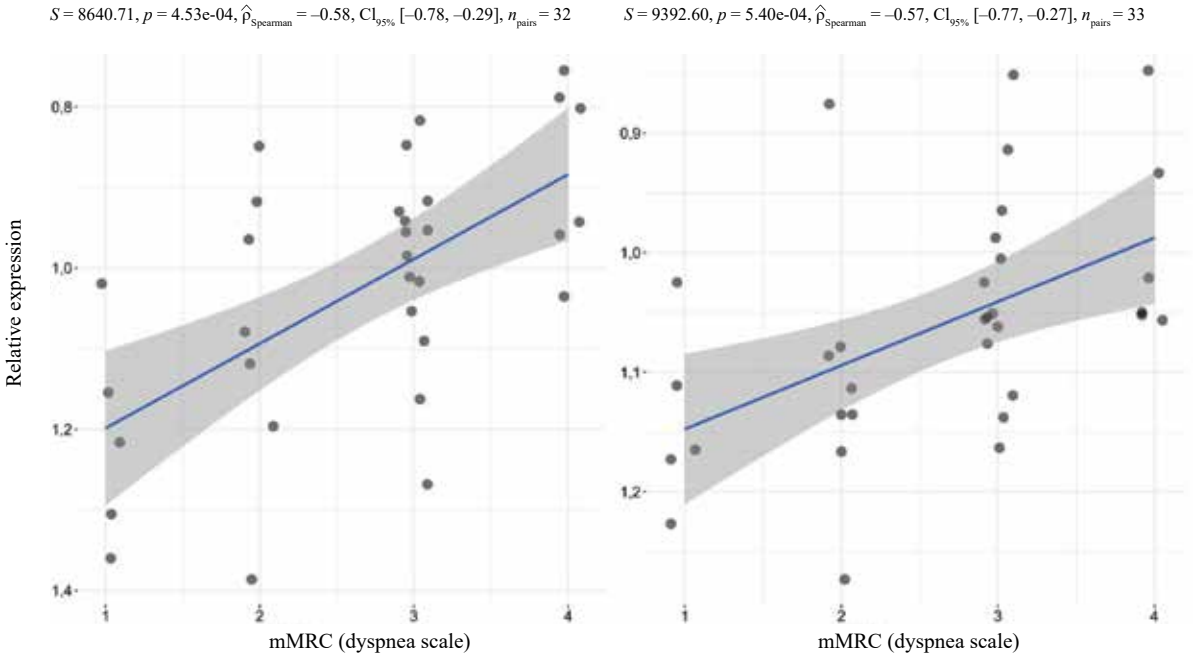


Fig. 2. Correlation between (*a*) TLR2 and (*b*) TLR6 gene expression level and the mMRC dyspnea scale

Table 2

**Allele frequencies of polymorphic TLR loci associated with the risk of development and severity of COPD**

| Locus | Allele | Groups | | Fisher's exact test, $p$ ($p_{Bonferroni}$) |
|---|---|---|---|---|
| | | COPD ($n$ = 103) | Control ($n$ = 47) | |
| rs5743551 (*TLR1*) | A | 167 (81%) | 73 (78%) | 0.5346 (1) |
| | G | 39 (19%) | 21 (22%) | |
| rs5743708 (*TLR2*) | A | 1 (0.5%) | 8 (9%) | **0.0005 (0.003)** |
| | G | 205 (99.5%) | 86 (91%) | |
| rs3804100 (*TLR2*) | C | 9 (4%) | 7 (7%) | 0.2781 (1) |
| | T | 197 (96%) | 87 (93%) | |
| rs4986790 (*TLR4*) | A | 187 (91%) | 88 (94%) | 0.5033 (1) |
| | G | 19 (9%) | 6 (6%) | |
| rs5743810 (*TLR6*) | A | 77 (37%) | 35 (37%) | 1 (1) |
| | G | 129 (63%) | 59 (63%) | |
| rs3764880 (*TLR8*) | A | 160 (78%) | 73 (78%) | 1 (1) |
| | G | 46 (22%) | 21 (22%) | |

Table 3

**Distribution of genotype frequencies in the COPD group by severity of the disease (according to FEV1)**

| Locus | Genotype | Severity of COPD according to FEV1 | | | | Fisher's exact test, $p$ ($p_{Holm-Bonferroni}$) |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | |
| rs5743810 (*TLR6*) | A/A | 0 (0%) | 7 (20%) | 6 (15%) | 1 (8%) | 0.034 (0.20*) |
| | A/G | 12 (75%) | 16 (46%) | 15 (39%) | 6 (46%) | |
| | G/G | 4 (25%) | 12 (34%) | 18 (46%) | 6 (46%) | |

* with correction for multiple comparisons (Holm-Bonferroni)

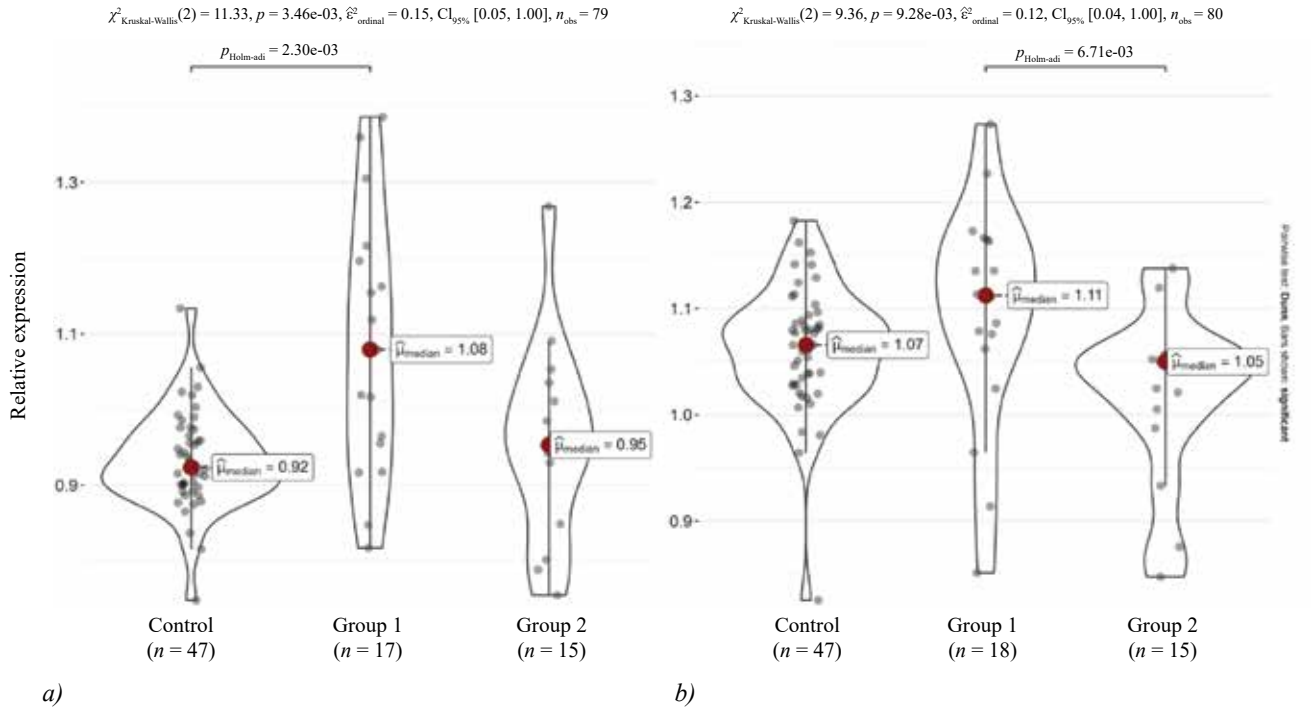


Fig. 3. Expression of TLR2 (*a*) and TLR6 (*b*) genes in the control group and in subgroups with mild and severe COPD according to the severity scale (GOLD) [1]

Considering that the studied indicators were part of the COPD severity assessment according to the severity scale (GOLD) [1], the COPD group was divided into subgroups according to COPD severity. Subgroups 1 and 2 were combined into a subgroup with mild/ moderate COPD, and subgroups 3 and 4 were combined into a subgroup with a more severe COPD. The results of the analysis are shown in Figure 3. There was an increased expression of *TLR2* and *TLR6* genes in patients with severe COPD and in the control group.

When the patients were divided according to the severity of COPD using the A, B, E scale (assessment of the FEV1 level, mMRC, and the frequency of exacerbations), only subgroups with mild and severe COPD significantly differed in the level of *TLR2* and *TLR6* gene expression.

## DISCUSSION

The absence of a statistically significant association between alleles and genotypes of the studied polymorphisms is not consistent with the previously obtained data on the associations of the studied alleles with other pathologies of the lower respiratory tract. In previous studies, the rs5743551-G (*TLR1*) and rs4986790-G (*TLR4*) alleles were identified to have an association with the risk of developing NETosis [19] – a marker of the inflammatory process severity. There was also an association discovered between the rs4986790-G (*TLR4*) allele and the risk of developing tuberculosis [20]. Although COPD exacerbations may be associated with the development of inflammation in the lungs caused by bacteria, earlier findings on the connection between the rs5743551-G (*TLR1*) allele and the development of acute bacterial infection in patients with pneumonia [11] were not confirmed in this study. Such a result may be explained by the predominance of non-bacterial reasons for COPD exacerbations in the study group. This is tied to the specific features of the disease: the main pathogenetic mechanism of COPD is a combination of modifiable environmental factors (smoking, ecology), that are a lot more influential than genetics. Viral infections are also a common factor for COPD exacerbations and the immunologic defense mechanisms to them are different from bacterial infections.

The findings in this study are consistent with those previously published; however, most studies have focused on the *TLR2* and *TLR4* genes. In the study published by S.E. Budulac et al., an association of FEV1 with several SNPs in the *TLR2* gene was identified, while the results for the rs5743708 and rs3804100 loci were not statistically significant [7]. Another study analyzed the contribution of the rs5743836 (*TLR9*) allele to the development of alveolar macrophage dysfunction and the progression of COPD [21].

Studying the connection between gene polymorphic variants and expression level allows not only to better

understand how genetic factors influence the emergence and the development of multifactorial diseases, but also how polymorphic variants affect gene functions. This study did not find an association between SNPs in TLR genes and changes in expression levels, but the observed trends towards increased expression levels may be explained by other factors. TLR molecular patterns activate not only in response to pathogens but also in response to lung tissue damage [22]. Since COPD development is usually associated with long-term exposure to cigarette smoke, COPD patients tend to have non-infectious damage to the lung tissue.

According to the literature, exposure to cigarette smoke can activate signaling cascades of DAMPs and oxidative stress [23]. Similar to TLR activation, oxidative stress is closely linked to interleukin production [24, 25]. Increased *TLR2* and *TLR6* expression levels, low blood oxygen saturation, and severe dyspnea may be associated with activation of oxidative stress and production of interleukins in response to smoking-induced lung tissue damage. High levels of interleukins may also contribute to decreased *TLR2* and *TLR6* gene expression in patients with COPD, which requires further studying.

## CONCLUSION

This study investigated the connection between polymorphic variants of TLR genes and the likelihood of COPD development and its course. No statistically significant association with rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) single nucleotide polymorphisms was found. The observed trend toward an increase in TLR gene expression may be associated with the remodeling of lung tissues and activation of the immune response by DAMPs that occur during COPD.

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