

ORIGINAL ARTICLE

SINGLE NUCLEOTIDE POLYMORPHISM RS4696480 OF *TLR2* GENE ASSOCIATES WITH SEVERITY OF ATOPIC DERMATITIS IN CHILDREN, BUT NOT WITH IGE SENSITIZATION TO MALASSEZIA

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ABSTRACT

The aim: Malassezia has been linked to atopic dermatitis, and TLRs are suggested to mediate influence of Malassezia spp on human cells. The aim of the study was to examine if *TLR2* rs4696480 polymorphism predisposes to atopic dermatitis, IgE sensitization to Malassezia or to severe phenotype of atopic dermatitis.

Materials and methods: The study included 103 patients with eczema and 84 healthy children. Specific IgE against Malassezia mix (m227) was analyzed in 47 patients using immunochemiluminiscent method on the ImmunoCAP 100 (Thermo Fisher Scientific Inc., Phadia, Sweden). Genotyping for *TLR2* rs4696480 was performed by using Real-time PCR.

Results: Increased IgE to Malassezia spp. was observed in 34,3 % of children with eczema. Higher Malassezia spp.-specific IgE titre positively correlated with duration of atopic dermatitis and a higher total IgE. There were no difference in allele distribution among patients and control group (OR=1.096 (0.549– 2.191) for AT, OR=0.946 (0.430– 2.078) for TT, $p > 0,05$). *TLR2* polymorphism rs4696480 was not associated with Malassezia spp.-sIgE. AA-genotype was significantly more frequent among patients with severe and moderate-to-severe AD (OR=6.395 (1.240–32.991)).

Conclusions: AA variant of *TLR2* rs4696480 polymorphism predisposes to severe phenotype of AD.

KEY WORDS: SNP; *TLR2*; Malassezia; atopic dermatitis; children

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INTRODUCTION

Atopic dermatitis (AD), the most common skin disease in infants and children, is a complex disease with a multifactorial etiology, including dysregulation of the immune system and dysfunction of the epidermal barrier, which are affected by both genetic and environmental factors [1].

Malassezia has been linked to a number of skin conditions, including AD. This is the most common fungal genus of healthy skin, but these yeasts also show pathogenic potential under appropriate conditions. The close relationship between skin and IgE-binding allergens in Malassezia in AD has been shown previously [2, 3]. They interact with almost all cellular components of the normal epidermis, including keratinocytes, Langerhans cells, melanocytes, and the host's immune system. It is known that Malassezia yeast is of great importance to the development of sensitization in AD. Malassezia colonizes human skin after birth, and therefore, as a commensal, must be normally recognized by the human immune system. The presence of SNPs in the PRR genes may be a prerequisite for the development of AD in children. Despite the current knowledge about the association of Malassezia species with the development of AD, the mechanisms underlying their change in their condition

from commensal to pathogenic still need to be further elucidated.

TLR are members of the PRR family, which recognize various preserved microbial components or PAMP. *TLR2* recognizes components of gram-positive bacteria and yeast, such as lipotechoic acid, peptidoglycan, lipoproteins or zymosan [2]. Recognition of PAMP by TLR initiates a signaling cascade that results in the production of proinflammatory cytokines, chemokines, antimicrobial peptides, and inducible enzymes in the skin by activating transcription factors, activator protein (AP) -1, and nuclear factor (NF) κ B [3-5]. TLRs are suggested to mediate influence of Malassezia on human cells. It has been discovered that *TLR2*, in particular, can recognize components of many yeasts including Malassezia [5]. Some studies conducted recently, confirmed the significance of TLRs for the immune response of human cells against Malassezia. For example, Malassezia induces the expression of *TLR2* and *TLR4* on human keratinocytes, which mediate the increased production of the antimicrobial peptide human beta-defensin 2 and the chemokine CXCL8. Others contribute to the pro-inflammatory response of dendritic cells against Malassezia to TLR-mediated mechanisms [6-8].

THE AIM

In the present study we investigate the frequencies of *TLR2* gene polymorphism T16934A (rs4696480) in children with AD to explore the association between the polymorphism and the sensibilization to *Malassezia* yeasts.

MATERIALS AND METHODS

The study included patients with AD ($n = 103$); aged 6 month to 18 years 6 [3;10] from department of allergy at Kyiv City Children Clinical Hospital №2 and 84 children aged 12 month to 18 years (6 [5;9]) without allergic disease at the time of examination or according to history data. This study was approved by the ethical committee of the O. Bogomolets national medical university, all patients/parents of affected children gave informed consent to participate.

OUTCOME MEASURES

The diagnosis of AD was established according to the criteria of Hanifin & Rajka [9], by the patient's history. Clinical parameters of patients including age, gender, age of onset and severity of eczema, AD distribution (exclusive head-neck type eczema vs diffuse AD including head and neck involvement), concomitant allergic diseases, parental history of atopy, total IgE and sIgE to *Malassezia*. Scoring of AD severity and blood sampling to determine IgE levels were performed at the same visit.

ASSESSMENT OF ATOPIC DERMATITIS SEVERITY

The severity of AD was assessed using SCORing Atopic Dermatitis (SCORAD) index. A SCORAD < 25 indicated mild AD, a SCORAD between 25 and 50 indicated moderate AD, and a SCORAD > 50 (with a maximum index of 103) indicated severe AD [10].

TESTING OF IGE ANTIBODIES

Specific IgE against *Malassezia* mix (m227), a mixture of 3 *Malassezia* species (*Malassezia sympodialis*, *Malassezia globosa*, and *Malassezia restricta*), was analyzed in 47 patients. Serum samples from patients were collected during visits and stored at -20°C until analysis. All samples were analysed together for the presence of total IgE antibodies and allergen-specific IgE antibodies to *Malassezia* spp. (m227) using immunochemiluminometric method on the ImmunoCAP 100 (Thermo Fisher Scientific Inc., Phadia, Sweden). The results were classified into classes: 0 (less than $0.35 \text{ kU \ l}$), 1 ($0.35\text{--}0.7 \text{ kU \ l}$), 2 ($0.7\text{--}3.5 \text{ kU \ l}$), 3 ($3.5\text{--}17.5 \text{ kU \ l}$), 4 ($17.5\text{--}50 \text{ kU \ l}$), 5 ($50\text{--}100 \text{ kU \ l}$) and 6 ($100 \text{ kU \ l}$). Class 1 or higher was defined as positive. The results of specific IgE to *Malassezia* were matched with patient-related parameters (gender, age, head-neck type eczema, SCORAD, age of onset, total IgE, concomitant allergic diseases).

SELECTION OF SNP

An SNP *TLR2* rs4696480 (A>T), which was located in intron region, was selected for genotyping, as it can affect

expression of *TLR2* and is reported to be common in European populations.

DNA EXTRACTION

Buccal epithelium was taken by using buccal brushes, skin samples were collected from the forearm by swabbing with the following freezing of samples and their storage at -20°C . DNA for genotyping was extracted from the samples by using NeoPrep 100 DNA (Neogen, Ukraine) according to manufacturer's protocol. The concentration of total DNA was determined by using a NanoDrop spectrophotometer ND1000 (NanoDrop Technologies Inc., USA).

qPCR GENOTYPING

Amplification reactions were performed by using a 7500 Fast Real-time PCR System ("Applied Biosystems", USA) in a final reaction volume of $20 \mu\text{l}$, which contained 2X TaqMan Universal Master Mix ("Applied Biosystems", USA), assay C_27994607_10 and the template DNA. The thermal cycling conditions involved a denaturation step at 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. Analysis of the data was carried out with 7500 Fast Real-Time PCR Software. The primer sequences of rs4696480 *TLR2* polymorphism were follows: For *TLR2*-F 5' AACAGAAATTTATCCATTCATGGTT 3', Rev *TLR2*-R 5' AGCAGTTTATTGTGAGAATGAGTTT 3' (<https://www.ncbi.nlm.nih.gov/SNP/>):

STATISTICAL ANALYSIS

Since the distribution of most of the sample characteristics differed from the Gaussian (normal) distribution, the statistical sample was heterogeneous, and therefore non-parametric statistical methods were used. Quantitative data for each of the study groups were presented as median - $\text{Me}[\text{QI}; \text{QIII}]$, categorical (dichotomous qualitative) variables - as the frequency of each of the values (n) and the percentage (%) in the group.

In the analysis of influence sensitization to *Malassezia* spp. on AD, Mann-Whitney U-test was used to compare differences between groups for quantitative data and Fisher's test was used for categorical variables. Spearman's ranking criterion (ρ) was used to assess the correlation between quantitative traits. A p -value < 0.05 was considered statistically significant.

Statistical processing was performed using EZR software version 1.32 (graphical interface R (version 2.13.0)).

SNPAnalyzer (web-based software) was used to examine Hardy-Weinberg equilibrium. χ^2 test was performed to investigate if there was any difference in the frequency of the genotype and the allele between the AD patient group and the healthy control group.

RESULTS

103 children between 6 months and 18 years of age were investigated in our study. The average age of children in

Table I. Characteristics of studied patients

Age, years, median (range)	6 [3;10]	
Male:Female	53:50	
SCORAD, median (range)	36,8 [30;60]	Minimum 0 Maximum 80
Categorised SCORAD, patients, %	35,9	
Mild (<25)	23,3	
Moderate (25–50)	20,5	
Severe (>50)	26,2	
Concomitant allergic diseases		
allergic rhinitis	32,0	
asthma	18,4	
allergic rhinitis+ asthma	14,6	
Early onset, %	85,0	
Sensitisation to <i>Malassezia</i> spp., %	34,3	

Table II. *TLR2* rs4696480 polymorphism genotype distribution among patients and control group and the association with clinical parameters of AD.

Clinical parameter	AA n (%)	AT n (%)	TT n (%)	p-values by OR
AD (n=103)	29 (28,1)	46 (44,7)	28 (27,2)	> 0,05
Controls (n=84)	23 (27,4)	40 (47,6)	21 (25,0)	
Severity by SCORAD				
Mild (n=23)	2 (8,7)	12 (52,2)	9 (39,1)	< 0,05
severe and moderate-to-severe (n=80)	27 (33,8)	34 (42,5)	19 (23,75)	
Age of onset				
early (before 18 mo) (n=86)	25 (29,1)	40 (46,5)	21 (24,4)	> 0,05
late (after 18 mo) (n=17)	4 (23,5)	6 (35,3)	7 (41,2)	
Asthma				
yes (n=16)	5 (31,3)	8 (50,0)	3 (18,7)	> 0,05
no (n=87)	24 (27,6)	38 (43,7)	25 (28,7)	
Allergic rhinitis				
yes (n=34)	10 (29,4)	15 (44,1)	9 (26,5)	> 0,05
no (n=69)	19 (27,5)	31 (44,9)	19 (27,5)	
Serum total IgE				
High total IgE (n=53)	12 (22,6)	23 (43,4)	18 (34,0)	> 0,05
Normal total IgE (n=50)	17 (34,0)	23 (46,0)	10 (20,0)	
sIgE to <i>Malassezia</i>				
yes (n=12)	4 (33,3)	6 (50,0)	2 (16,7)	> 0,05
no (n=35)	9 (25,7)	15 (42,9)	11 (31,4)	
Parental history of atopy				
yes (n=37)	11 (29,7)	16 (43,2)	10 (27,0)	> 0,05
no (n=66)	18 (27,3)	30 (45,5)	18 (27,3)	

the main group was 6 [3;10] years, including 53 boys and 50 girls. The clinical characteristics of patients are summarised in Table I.

Serum IgE to *Malassezia* was evaluated in 47 children. Increased IgE to *Malassezia* spp. was observed in 12 (34,3%) children with AD. Among them 4 (33,3 %) children had class 1 of sensitization, 3 (25,0 %) children had 3rd class,

5 (41,7 %) children – the 4th class. Among children with positive IgE to *Malassezia*, there were 6 male and 6 female ($p > 0.05$).

We have found a relationship between higher *Malassezia* spp.-specific IgE titre and a higher total IgE and the age of the onset, but not the severity and the duration of eczema. Data are presented on Figures 1-4.

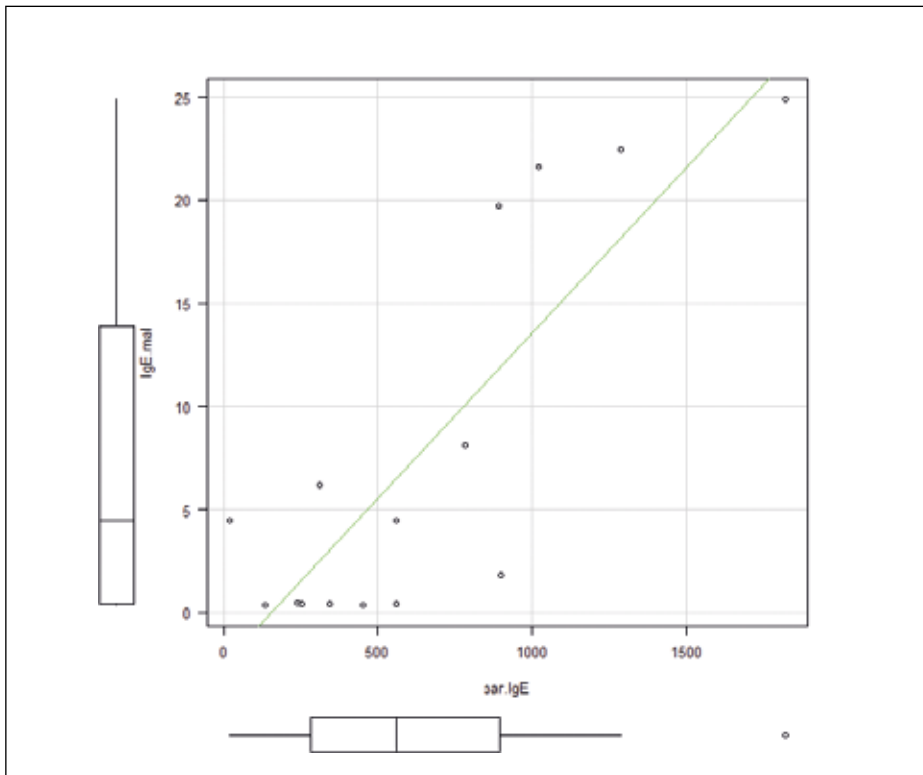


Fig. 1. Correlation of total IgE and Malassezia spp.-specific IgE, $p=0,00625$

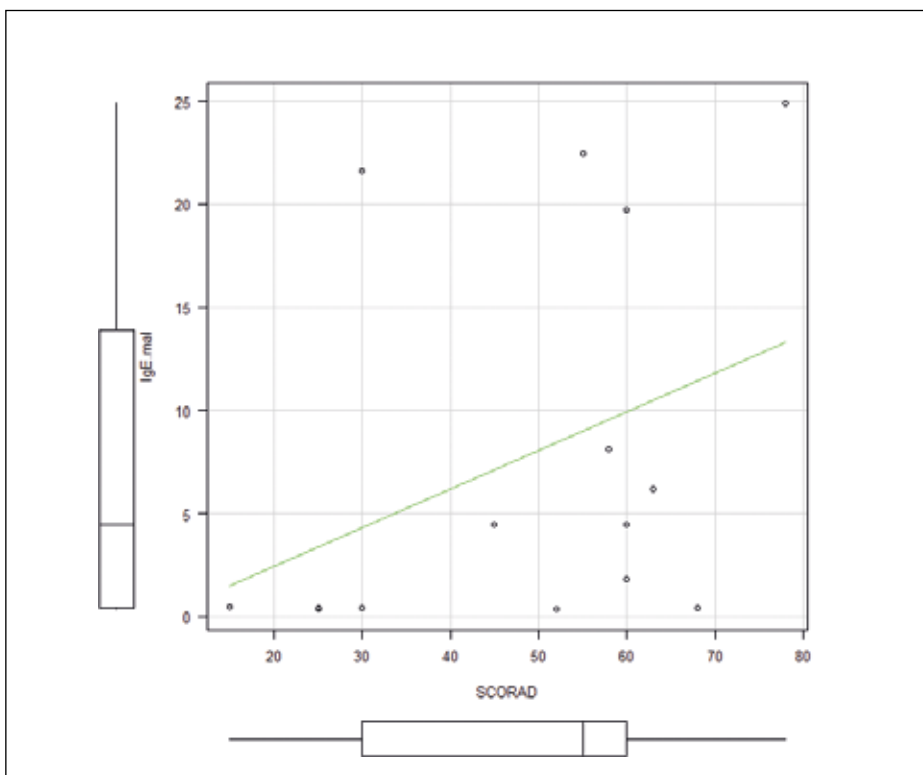


Fig. 2. Correlation of SCORAD and Malassezia spp.-specific IgE, $p=0,133$

GENOTYPING

We found that SNP was in Hardy-Weinberg equilibrium. There was no difference in genotype distribution among patients and control group (OR=1.096 (0.549-2.191) for AT, OR=0.946 (0.430– 2.078) for TT, $p > 0,05$) (Table II).

We investigated the association of the *TLR2* rs4696480 polymorphism with clinical parameters of AD (severity on

the SCORAD scale, age of the onset, presence of concomitant allergic diseases, serum total IgE level, presence of sIgE to *Malassezia*, the parental history of the atopy). AA-genotype was significantly more frequent among patients with severe and moderate-to-severe AD (OR=6.395 (1.240-32.991) (Figure 5). Other clinical parameters and features of the phenotype were not related to the genotype of patients (Table II).

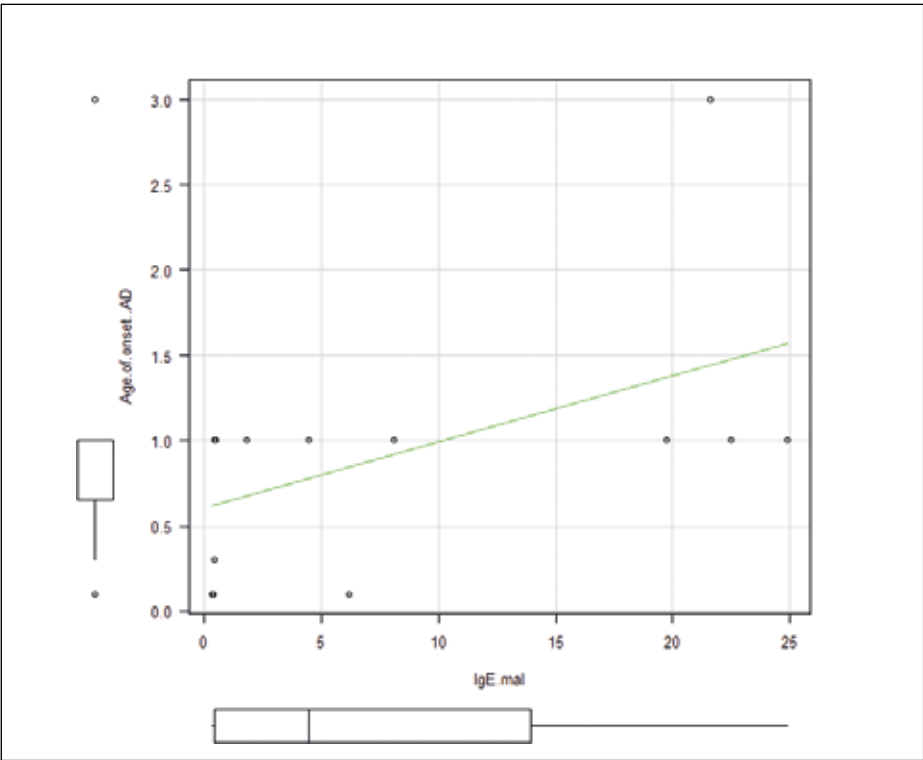


Fig. 3. Correlation of age of onset of AD and *Malassezia* spp.-specific IgE, $p=0,0372$.

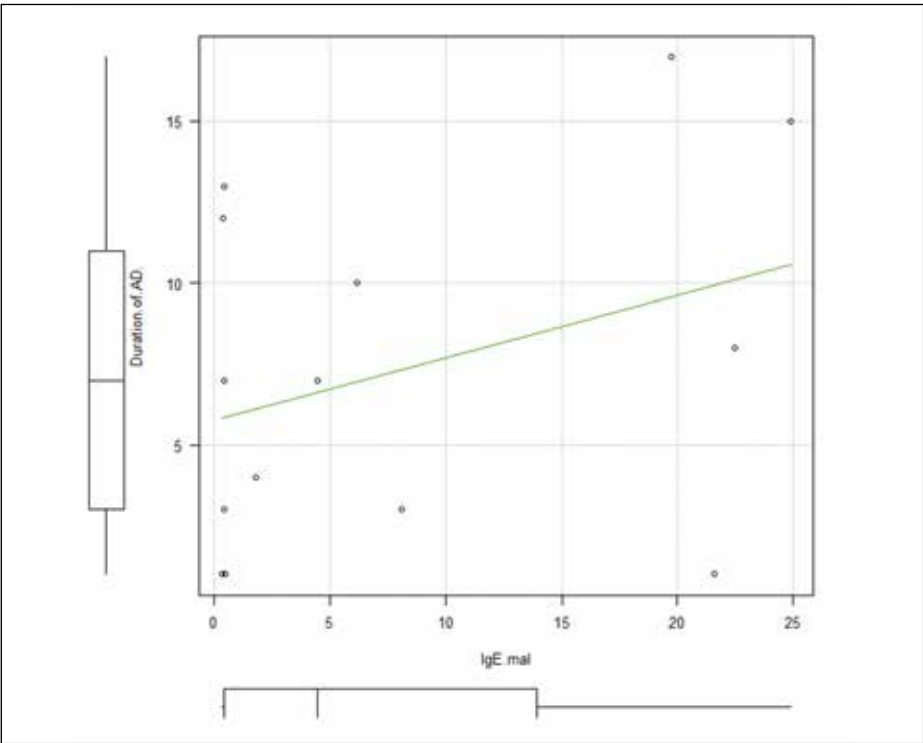


Fig. 4. Correlation of duration of AD and *Malassezia* spp.-specific IgE, $p=0,375$.

DISCUSSION

TLRs play the key role in the innate immune system and are directly engaged in the pathogenesis of many skin diseases as they recognize molecules of many pathogens. The Salpietro C. study identified the overall variant *TLR2* rs4696480 as the most common in children with AD using the candidate gene method in a cohort of 187 children with AD and 150 healthy controls [11]. SNP rs4696480 in *TLR2*

is located near an evolutionarily conserved sequence as well as several transcription factor binding sites, including retinoid receptors, NF- κ B, and the interferon regulatory factor [12]. The presence of multiple transcription factor binding sites in an evolutionarily conserved sequence indicates that this region is transcriptionally relevant and potentially is genetically or transcriptionally reflected. The Oh D.Y. study reported an increase in the incidence of A-allele at position

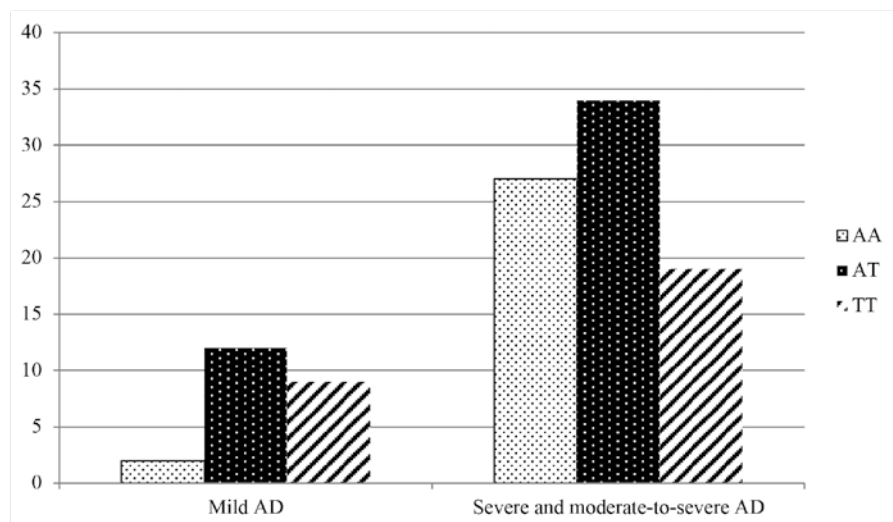


Fig. 5. TLR2 rs4696480 and severity of AD according to SCORAD

rs4696480 of the *TLR2* gene among German adult patients with severe AD (SCORAD > 50) as compared to patients with mild-to-moderate AD (SCORAD ~ 50) [12]. Eder et al. [13] reported an association between the *TLR2* rs4696480 SNP gene and asthma, wheezing, atopy, or symptoms of allergic rhinitis in children of farmers. It has been suggested that the T allele in the promoter region of the *TLR2* gene has a protective effect against these diseases. Other research groups have found an inverse association between polymorphism and AD severity: Polymorphism in *TLR2* rs4696480 was associated with SCORAD severity, asthma, allergic conjunctivitis, or family history of atopy in patients with AD with serum IgE ≥ 106 IU / ml, but not in those with IgE < 106 IU / ml [14]. Our study has not revealed difference in genotype distribution among Ukrainian children with eczema and healthy controls. Therefore, the influence of polymorphism rs4696480 on AD susceptibility can be population-dependent.

Among clinical features, the severity of AD was related to the genotype. AA-genotype was significantly more frequent among patients with increased severe and moderate-to-severe AD (OR=6.395 (1.240-32.991). TLRs are suggested to mediate the interaction between *Malassezia* cells (or their immunogenic proteins) and human cells. It is not fully understood if *Malassezia* sensitization can be mediated via TLR2, and how functional SNPs can contribute to the process of sensitization. Selander C. et al. determined that *M. sympodialis* can activate mast cells, enhance the mast cell IgE response, modulate MAPK activation, and by signaling through the TLR2/MyD88 pathway alter IL-6 production in a dose-dependent manner [15]. In our study genotype did not correlate with *Malassezia* spp.-sIgE, but the study group was small.

CONCLUSIONS

Malassezia spp. plays an important role in AD, as it can interact with the skin immune system. The impaired skin barrier of atopic individuals and sensitisation to *Malassezia* spp. correlates with the severity of AD. AA variant

of *TLR2* rs4696480 polymorphism predisposes to severe phenotype of AD.

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Conflict of interest:

The Authors declare no conflicts of interest.

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