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Polymorphisms of Toll-Like Receptor 4 as a Risk Factor for Periodontitis: Meta-Analysis

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

Abstract

Background. Toll-like receptors play an important role in the recognition of periopathogens. There are widely known polymorphisms of these receptors that may alter host susceptibility to periodontitis.

Objectives. Evaluating the association of TLR4 polymorphisms (Asp299 and Thr399Ile) with the occurrence of periodontitis and its two clinical types: chronic (CP) and aggressive (AgP) among Caucasians.

Material and Methods. A review of 4 electronic databases was made to identify studies that match the inclusion criteria for the meta-analysis and were published up to December 31, 2013.

Results. Fifteen studies met the inclusion criteria. For Asp299Gly polymorphism 1621 patients with periodontitis (1148 with CP and 473 with AgP) and 1755 individuals without clinical signs of periodontitis were included in the meta-analysis. The occurrence of polymorphism Thr399Ile was evaluated among 1522 patients with periodontitis (1017 with CP and 505 with AgP) and 1461 subjects in the control group. The pooled OR with a random effects model for the Asp299Gly and chronic periodontitis was 1.35 (95% CI: 1.02–1.8, $p = 0.038$), indicating a possible association between this polymorphism and CP. Other published meta-analyses showed no significant association between single nucleotide polymorphisms of TLR4 and periodontitis nor its clinical types. No publication bias was reported.

Conclusions. Our meta-analysis showed statistically significant association between TLR4 Asp299Gly allele and increased susceptibility to chronic periodontitis (*Adv Clin Exp Med* 2015, 24, 6, 1059–1070).

Key words: toll-like receptor, polymorphisms, periodontitis, meta-analysis.

Periodontitis is an infectious and inflammatory disease, which when untreated, leads to progressive destruction of the teeth supporting tissues and, in consequence, loss of teeth. The imbalance between periopathogens and host factors (determined in about 50% by the genetics) is responsible for the transition of gingivitis (early, completely reversible stage) into periodontitis.

The pathophysiology of periodontitis includes interactions between genetic predisposition dependent on many genes and environmental factors (Gram-negative periopathogens, smoking, socio-economic status). This leads to the development of two phenotypic types – more frequent chronic periodontitis (CP) and very rare, progressing faster aggressive periodontitis (AgP) [1–3]. Since 1997 [4] researchers have been trying to identify genetic mutations responsible for host

susceptibility to periodontal disease in different populations. So far for the Caucasians in the meta-analysis the composite genotype of IL-1A (C[–889] T/IL-1B (C[3953/4]T) in chronic periodontitis has demonstrated the strongest correlation (OR: 2.25; CI: 1.21–4.12) [5].

Toll-like receptors (TLRs) are a family of transmembrane proteins expressed by immunologically competent cells. They recognize and bind pathogen associated molecular patterns (PAMPs), derived from bacteria, fungi, viruses and protozoa. Each type of receptor recognizes and binds specific ligands, initiating a cascade of signaling pathways through MyD88-dependent or TRIF-dependent manner. This leads to secretion of NF- κ B, which enters the nucleus of the cell and induces expression of inflammatory cytokines, chemokines, adhesion molecules, growth factors and interferons [6, 7].

Biological function of TLRs (Toll-like receptors) in infection control makes them good candidates in the context of their involvement with genetic risk factors for periodontitis.

TLR4 is present on antigen presenting cells, fibroblasts and keratinocytes of gingival epithelium. To recognize PAMPs of periopathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Veillonella parvula* TLR4 cooperates with CD14 protein and MD2 complex, binding lipid A (endotoxin) of lipopolysaccharide [8, 9]. Repeated stimulations of these receptors lead to development of tolerance to bacterial products [10]. Single nucleotide polymorphisms (SNPs) of TLR4 genes may change it.

TLR4 is located on chromosome 9q32-q33. For Caucasians two co-segregated polymorphisms were identified, resulting in structural changes in the extracellular domain of this receptor. First SNP results in substitution of aspartic acid with glycine at position 299 (Asp299Gly, 896A/G, SNP ID: rs4986790) and second one substitution of threonine with isoleucine at position 399 (The399Ile, 1196 C/T, SNP ID: rs4986791) [11]. These polymorphic alleles occur at frequency of more than 5% (e.g. in Canadian Caucasians 10%, Caucasian Americans 6.3 and 5.3%, Germans 5.6 and 6%, Belgians 8%) [12]. The distribution of these mutations is explained by the differences in the pressure of environmental pathogens on human populations which result in their specific geographical spread. For example, in Europe Asp299Gly and The399Ile commonly co-segregate where in Africa Asp299Gly/wild-type genotype occurs more often [13] and their functional consequences have not been completely elucidated.

There are few European case-control studies which demonstrate significant association of Asp299Gly polymorphism with increased risk of endotoxemia, urinary tract infection in children and meningococcal disease in infants. The399Ile polymorphism is linked to greater risk of developing hematogenous osteomyelitis and tonsillar disease associated with *Streptococcus pyogenes* [11, 13, 14]. Conducted meta-analysis showed significant association of TLR4 polymorphisms Asp299Gly only with parasitic infections (8 trials, OR – 1.59; CI – 1.05–2.42; $p < 0.001$) [14].

Immunohistochemical studies revealed the presence of TLR1–TLR9 in gingival epithelium and the subepithelial connective tissue of healthy gingiva. Significant increase in the expression ($p < 0.0001$) of all of these receptors was reported in the course of periodontitis [15]. Kinane et al. [16] in an *in vitro* trial stimulated epithelial gingival cells obtained from subjects who are heterozygotes for Asp299Gly and The399Ile with LPS

derived from *Porphyromonas gingivalis*. They reported that those cells were hyporeactive in secretion of proinflammatory cytokines. It remains to be elucidated whether this functional effect has phenotypic consequences, or in other words if there is an association between polymorphisms of TLR4 gene identified among Caucasians and susceptibility to periodontitis.

In this study, meta-analysis was used to determine correlations between both polymorphisms of TLR4 gene and occurrence of periodontitis and its two clinical types in previous conducted clinical trials for Caucasian populations.

Material and Methods

A systematic review of the literature was performed for articles published up to December 31, 2013, taking into consideration the following electronic databases: PubMed, Embase, Scopus, and the Polish Medical Bibliography. Phrases like “TLR4”, “Toll-Like Receptor 4” linked up to “periodontitis”, “periodontal disease” and “polymorphism” were used. Studies published in English, German, Russian and Polish were taken into account. The search was carried out independently by the two authors (TK, DC). PRISMA guidelines for systemic reviews and meta-analyses were used [17].

The first selection brought 42 results (identification). Additionally, all references in these articles were verified to find studies that were not included in electronic databases. Reviews, papers with others than examined polymorphisms of TLR4 gene and with incomplete genotype were rejected (screening).

The following inclusion criteria for the eligibility of full-text articles were adopted:

- 1) case-control trials,
- 2) periodontitis defined as chronic or aggressive,
- 3) caucasians as a study population,
- 4) only one (most numerous) study by the same group of authors was included,
- 5) technology of genotyping (SNP cluster ID: rs 4986790 or rs 4986791).

The following exclusion criteria for full-text articles were adopted:

- 1) absence of clear identification of study (type of periodontitis) and control groups,
- 2) significant demographic differences between study and control groups,
- 3) other than Caucasian race that was included in the study,
- 4) absence of adequate information about genotyping technology.

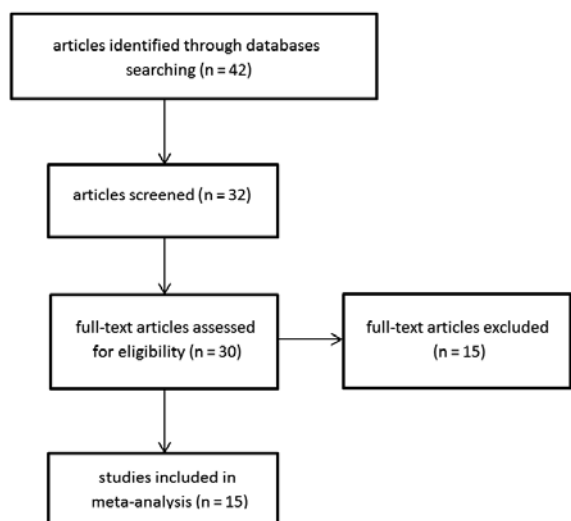


Fig. 1. PRISMA flow diagram

Selecting process of studies is illustrated on PRISMA flow Diagram (Fig. 1).

From obtained publications the following information was recovered: author, year of publication, number of test and control groups, number of subjects who are heterozygotes or homozygous for Asp299Gly and Thr399Ile in both groups and the distribution of polymorphic and wild types alleles in both groups. The general characteristics of the studies included in the meta-analysis are summarized in Table 1.

Statistical Analysis

From the publications selected for the meta-analysis, the frequency differences of polymorphic alleles between patients diagnosed with periodontitis, chronic periodontitis (CP) or aggressive periodontitis (AgP) and the control group were calculated using the chi-square test. The Hardy-Weinberg Equilibrium was also evaluated using the chi-square test by means of the calculator for biallelic markers (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). For the calculation of the cumulative OR random effects model was used in the method of DerSimonian-Liard. Homogeneity of the studies included in the meta-analysis was examined using Cochran's Q and I^2 tests. To assess the publication bias, Egger's test was used and funnel plot was created. The significance level for all tests within the meta-analysis was established as $p < 0.05$.

The analysis was carried out using STATISTICA 10.0 with Medical Kit 2.0.

Results

Studies Included in the Meta-Analysis

Fifteen studies met the inclusion criteria [18–32]. Two of these studies [27, 31] evaluated only Asp299Gly polymorphism and one study evaluated only Thr399Ile polymorphism. The rest of the publications included 2 polymorphisms. In 8 articles [18, 21, 23, 25–27, 30, 31] study groups consisted of subjects with chronic periodontitis, in 2 [24, 29] aggressive periodontitis and in 5 [19, 20, 22, 28, 32] both clinical types of this disease were analyzed. The distribution of wild type and polymorphic alleles in all control groups (in relation to periodontitis defined as one diagnosis and to its clinical types CP and AgP) was consistent with Hardy-Weinberg Equilibrium except for Turkish studies by Emingil et al. [24] (Table 1).

For Asp299Gly polymorphism 1621 individuals with periodontitis (CP 1148 and AgP 473) and 1755 patients without clinical signs of periodontitis were evaluated. Most of these studies were conducted among the Germans [18, 19, 26, 28–30] – 1355 people, then the British [20, 22] – 580 people, the Turks [23, 24] – 434 people, the Czechs [25] – 389 people, the Finns [27] – 229 people, the Dutch [21] – 199 people and the Americans [31] – 190 people. The frequency of Asp299Gly polymorphism was 12.83% among subjects with periodontitis (13.76% in CP and 10.57% in AgP) and 12.14% in the control group. The frequency of Asp299Gly allele in periodontitis was 6.44% (6.93% in CP and 5.28% in AgP) and 5.47% in the control group. Significantly higher prevalence of Asp299Gly was shown only for CP ($p = 0.0385$) (periodontitis ($p = 0.071$), AgP $p = 0.588$)). Almost all subjects were heterozygotes for Asp299Gly. Only James et al. [22] identified one homozygous individual in CP and three studies described such patients in the control groups [21, 24, 31].

The Thr399Ile polymorphism was evaluated in a total of 1,522 patients with periodontitis (1,017 with CP and 505 with AgP) and 1,461 in the control group. These studies were conducted mostly among the Germans [18, 19, 26, 28–30] – 1,435 people and then the British [20, 22] – 675 people, the Turks [23, 24] – 434 people, the Czechs [25] – 389 people, the Dutch [21] – 199 people and the Poles [32] – 92 people. The Thr399Ile polymorphism frequency in patients with periodontal disease was 11.82% (12.59% with CP and 10.3% with AgP) and 10.2% for all subjects in the control group. The frequency of Thr399Ile allele in all individuals diagnosed with periodontitis was 6.32% (CP 6.34% and AgP 5.15%) and 5.41% in the control group.

Table 1. Characteristics of the case-control studies included in the meta-analysis

Author, year, country	case N	cont. N	RR	Asp299Gly OR 95%CI random	W (%)	MAF case	MAF control	Thr399Ile OR 95%CI random	W (%)	MAF case	MAF cont.	HWE p
Chronic periodontitis												
Folwaczny et al. 2004 Germany	122	122	–	1.27 0.48–3.34	7.47	0.04	0.03	1.24 0.5–3.12	10.61	0.05	0.04	0.708 0.672
Schröder et al. 2005 Germany	116	116	+	4.78 1.88–12.2	7.86	0.1	0.03	5.04 1.98–12.8	10.44	0.11	0.03	0.775 0.775
Brett et al. 2005 England	53	97	–	1.64 0.52–5.16	5.55	0.06	0.04	0.37 0.12–1.15	8.19	0.04	0.09	0.712 0.338
Laine et al. 2005 Netherland	100	99	–	1.11 0.43–2.86	7.72	0.05	0.05	1.11 0.43–2.86	10.27	0.05	0.05	0.117 0.117
James et al. 2007 England	95	94	–	1.14 0.54–2.4	11.4	0.1	0.09	1.18 0.58–2.4	13.56	0.12	0.1	0.367 0.298
Berdeli et al. 2007 Turkey	83	106	–	0.84 0.23–3.09	4.44	0.02	0.03	0.76 0.18–3.27	5.84	0.02	0.02	0.764 0.803
Hola et al. 2007 Czech Republic	171	218	–	1.38 0.75–2.55	15.1	0.07	0.05	1.45 0.79–2.69	15.02	0.07	0.05	0.41 0.432
Reismann et al. 2007 Germany	75	54	–	4.0 0.84–19.1	3.16	0.07	0.02	4.0 0.84–19.1	5.29	0.07	0.02	0.889 0.889
Tervonen et al. 2007 Finland	51	178	–	1.35 0.65–2.8	11.7	0.13	0.1	n.d.	n.d.	n.d.	n.d.	0.133 n.d.
Schulz et al. 2008 Germany	60	80	–	1.6 0.55–4.7	6.21	0.07	0.04	1.6 0.55–4.7	8.87	0.07	0.04	0.682 0.682
Noack et al. 2009 Germany	108	76	–	1.07 0.41–2.74	7.71	0.06	0.05	1.07 0.41–2.74	10.27	0.06	0.05	0.628 0.628
Sahingur et al. 2011 USA	114	76	–	0.69 0.33–1.44	11.7	0.08	0.12	n.d.	n.d.	n.d.	n.d.	0.942 n.d.
Chrzesczyk 2013 Poland	34	30	–	n.d.	n.d.	n.d.	n.d.	0.16 0.01–3.58	1.65	0	0.03	n.d. 0.85
Aggressive periodontitis												
Schroder et al. 2005 Germany	81	81	–	2.47 0.9–5.59	16.6	0.1	0.05	2.47 0.9–5.59	16.26	0.1	0.05	0.64 0.64
Brett et al. 2005 England	45	97	–	2.78 0.94–8.22	14	0.09	0.04	0.3 0.08–1.07	13.01	0.03	0.09	0.712 0.338
James et al. 2007 England	73	123	+	0.29 0.09–0.91	13.6	0.03	0.08	0.29 0.09–0.91	14.32	0.03	0.08	0.326 0.326
Emingil et al. 2007 Turkey	90	155	–	0.85 0.25–2.92	12.2	0.02	0.03	0.48 0.1–2.36	10.64	0.01	0.02	0.013 0.773
Schulz et al. 2008 Germany	73	80	–	1.11 0.37–3.32	13.8	0.05	0.04	1.11 0.37–3.32	14.57	0.05	0.04	0.682 0.682
Noack et al. 2008 Germany	111	80	–	0.86 0.34–2.2	16.3	0.05	0.06	0.86 0.34–2.2	16.08	0.05	0.06	0.593 0.593
Chrzesczyk 2013 Poland	28	30	+	n.d.	n.d.	n.d.	n.d.	6.63 1.29–34.2	10.33	0.16	0.03	n.d. 0.85

RR – reported results; + positive association; – no association; W – study weight; MAF – rare allele frequency; OR (95% CI) calculated in own meta-analysis under random-effects model; n.d. – no data; HWE p – Hardy-Weinberg equilibrium.

Significantly higher prevalence of this allele was shown in chronic periodontitis ($p = 0.0495$) and the absence of significant results for people with a general diagnosis of periodontitis ($p = 0.156$) and for the AgP group ($p = 0.755$) was reported. Only two homozygous subjects were found, one in the CP [22] and one in the control group [21].

Meta-Analysis of the TLR4 Asp299Gly Polymorphism and Susceptibility to Periodontitis

The pooled OR with a random effects model for clinical diagnosis of periodontitis was 1.26 (95% CI: 0.95–1.69; $p = 0.11$) (Fig. 2). The variance of the real effects T^2 was 0.096. Only one study [19]

demonstrated a significant association between the presence of Asp299Gly and increased risk of periodontitis. There was no heterogeneity of the studies included in the meta-analysis (the Cochran Q test: 18.71; $p = 0.09$ and I^2 35.87%; 95% CI: 0.0–66.82%) and there was also no significant publishing bias detected (the Egger test: $p = 0.67$) (Fig. 3).

The pooled OR with a random effects model for the Asp299Gly and chronic periodontitis (fixed effect data not shown, the variance of the real effects T^2 : 0.045) was 1.35 (95% CI: 1.02–1.8; $p = 0.038$) (Fig. 4), which indicates a possible link between presence of polymorphic alleles and the occurrence of chronic periodontitis. The omission of American [31] and Turkish [23] studies with the least certain homogeneity, enhanced the value

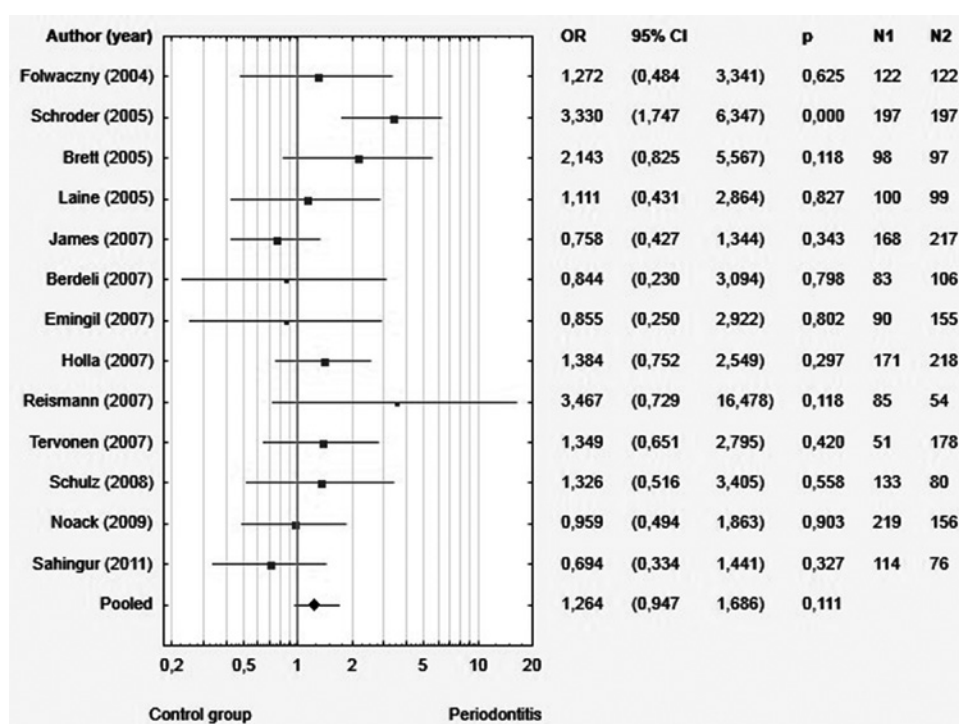


Fig. 2. Forest plot of published case-control studies of Asp299Gly TLR4 in periodontitis

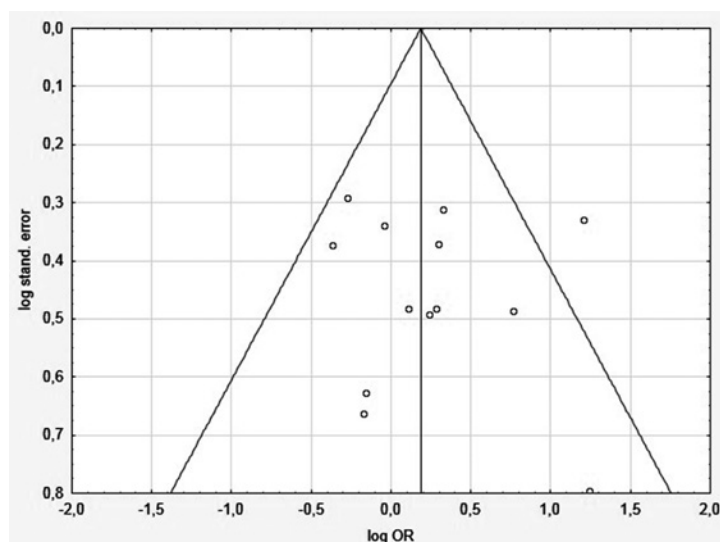


Fig. 3. Funnel plot of Asp299Gly TLR4 in periodontitis studies included in the meta-analysis. Value of Egger's linear regression test: $R = 0.51$; $p = 0.67$

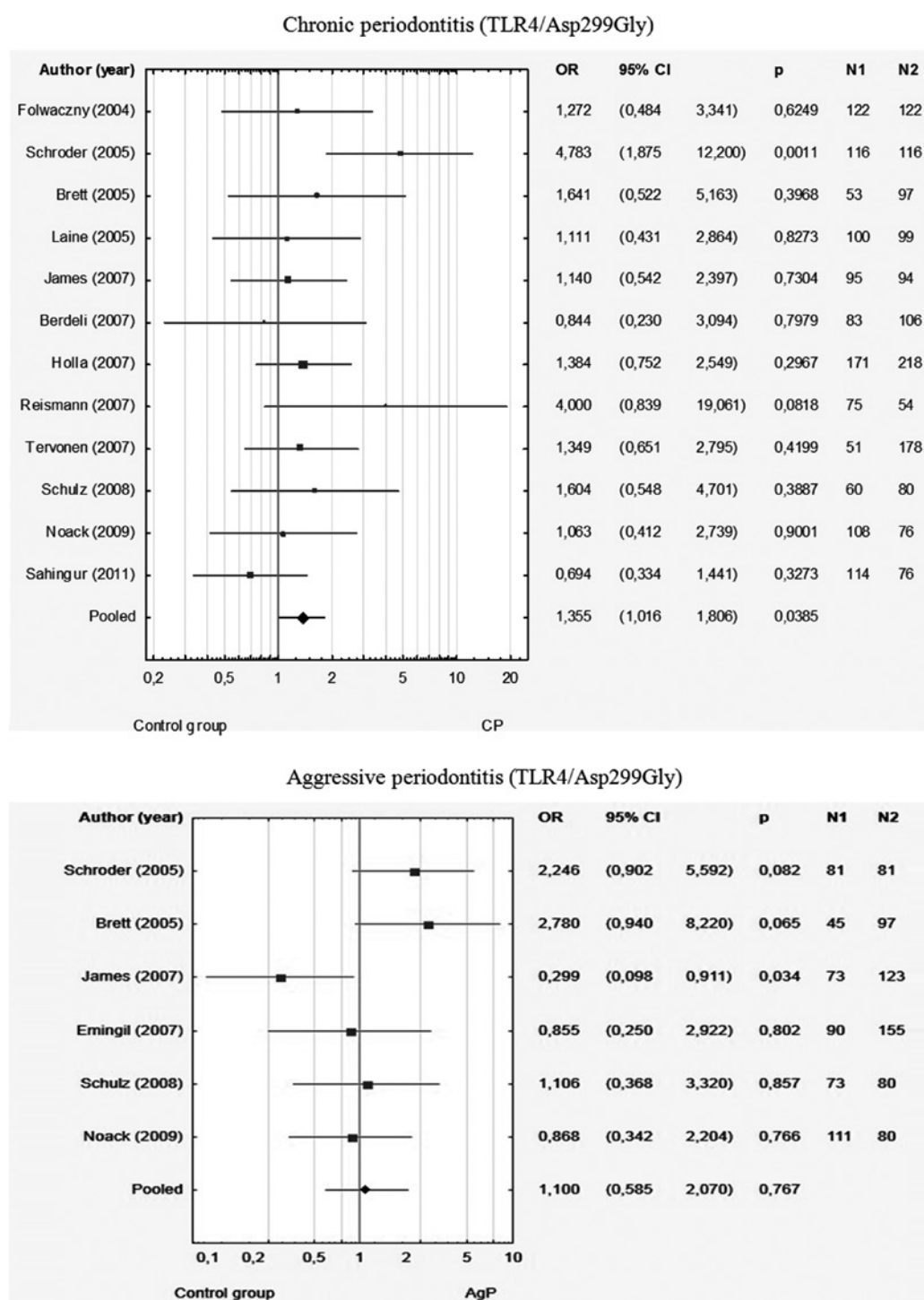


Fig. 4. Forest plot of published case-control studies of Asp299Gly TLR4 in CP and AgP

of the pooled OR, which for only German, English, Dutch, Czech and Finnish trials was 1.51 (95% CI: 1.13–1.99; $p = 0.004$). There was no heterogeneity in 12 studies included in this meta-analysis (the Cochran Q test: 13.39; $p = 0.27$ and I^2 17.85%; 95% CI: 0.0–57.14%) and no significant publishing bias (the Egger test: $p = 0.29$).

The value of pooled OR does not show a significant association between Asp299Gly and aggressive periodontitis (OR: 1.01; 95% CI: 0.58–2.07, $p = 0.77$) (Fig. 4). The variance of the real effects T^2 for this meta-analysis was 0.333. There was no

heterogeneity detected in 6 included studies (Cochran Q test value: 10.8; $p = 0.055$ and I^2 53.7%; 95% CI: 0.0–81.48%) and there was no significant publication bias (the Egger test: $p = 0.49$).

Meta-Analysis of the TLR4 Thr399Ile Polymorphism and Susceptibility to Periodontitis

There was no significant association between the presence of the Thr399Ile allele and clinical diagnosis of periodontitis (OR: 1.18; 95% CI:

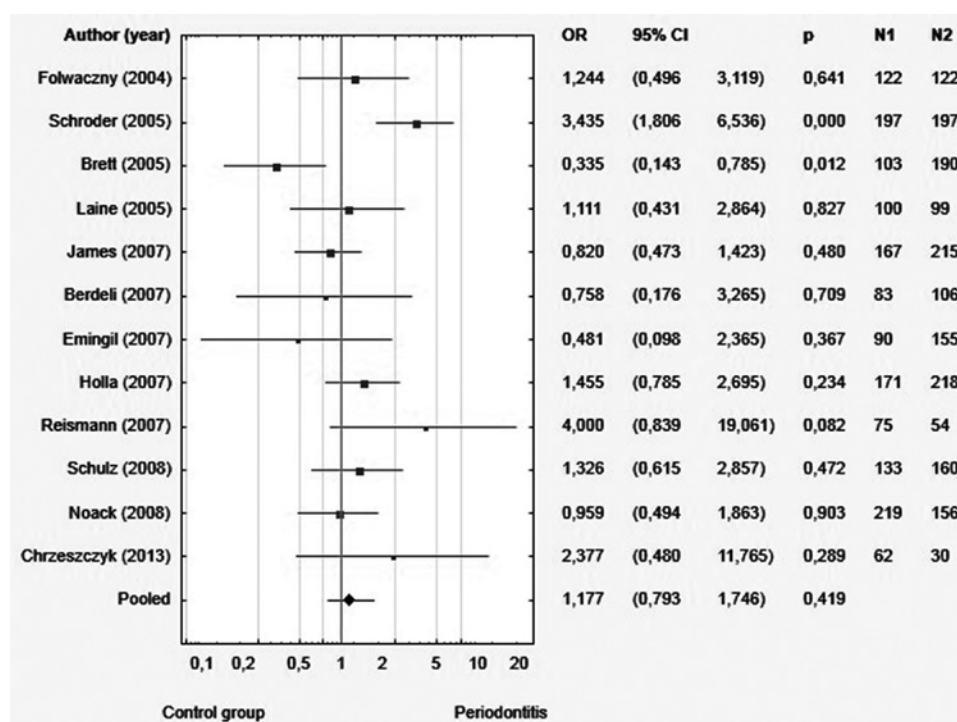


Fig. 5. Forest plot of published case-control studies of Thr399Ile TLR4 in periodontitis

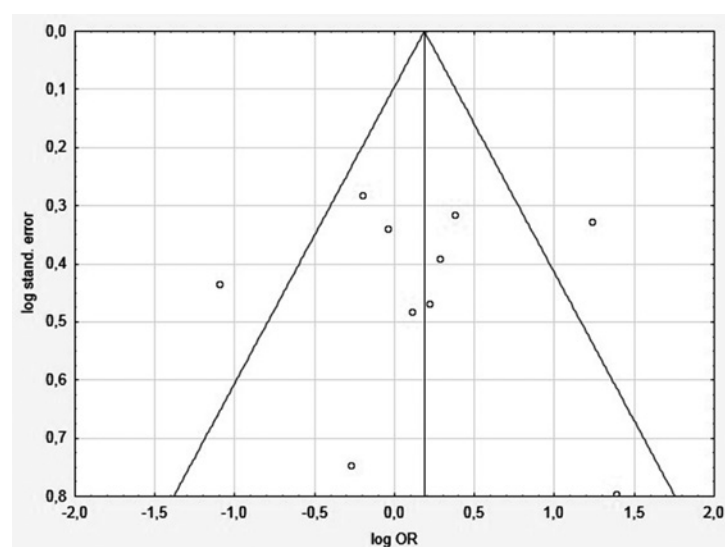


Fig. 6. Funnel plot of Thr399Ile TLR4 in periodontitis studies included in the meta-analysis. Value of Egger's linear regression test: $R = 0.09$; $p = 0.94$

0.79–1.75; $p = 0.42$) (Fig. 5). The variance of the real effects T^2 was 0.25. Two studies found a significant but contradictory association with this genotype [19, 20]. The heterogeneity of the studies was observed (Cochran Q test: 26.27; $p = 0.006$ and I^2 58.13%; 95% CI: 20.59–77.92%) and no publication bias was detected (the Egger test: 0.94) (Fig. 6).

The pooled OR for the association between this allele and chronic periodontitis in a random effects model (the variance of the real effects $T^2 = 0.19$) was 1.32 and was not statistically significant (CI: 0.87–1.98; $p = 0.18$) (Fig. 7). Of the 11 analyzed studies only Schröder et al. [19] showed significantly higher frequency of Thr399Ile polymorphism in CP. Stratification by removing uncertain in terms of racial factor Turkish studies [23] did

not affect the value of the pooled OR. There was no heterogeneity of the studies included in the meta-analysis (the Cochran Q test: 17.58; $p = 0.062$ and I^2 : 43.61%, 95% CI: 0.0–71.88%). No publication bias was observed (the Egger test: $p = 0.29$).

The pooled OR in a random effects model (the variance of the real effects $T^2 = 0.63$) showed no association between the genotype and the prevalence of aggressive periodontitis (OR: 0.91; CI: 0.43–1.92; $p = 0.8$) (Fig. 7). James et al. [22] reported significantly lower and Chrzęszczyk [32] significantly higher frequency of Thr399Ile in AgP. The heterogeneity of the studies included in this meta-analysis was observed (Cochran Q test: 16.88; $p = 0.09$ and I^2 : 64.45%; 95% CI: 19.86–84.23%) and there was no significant publication bias (the Egger test: $p = 0.84$).

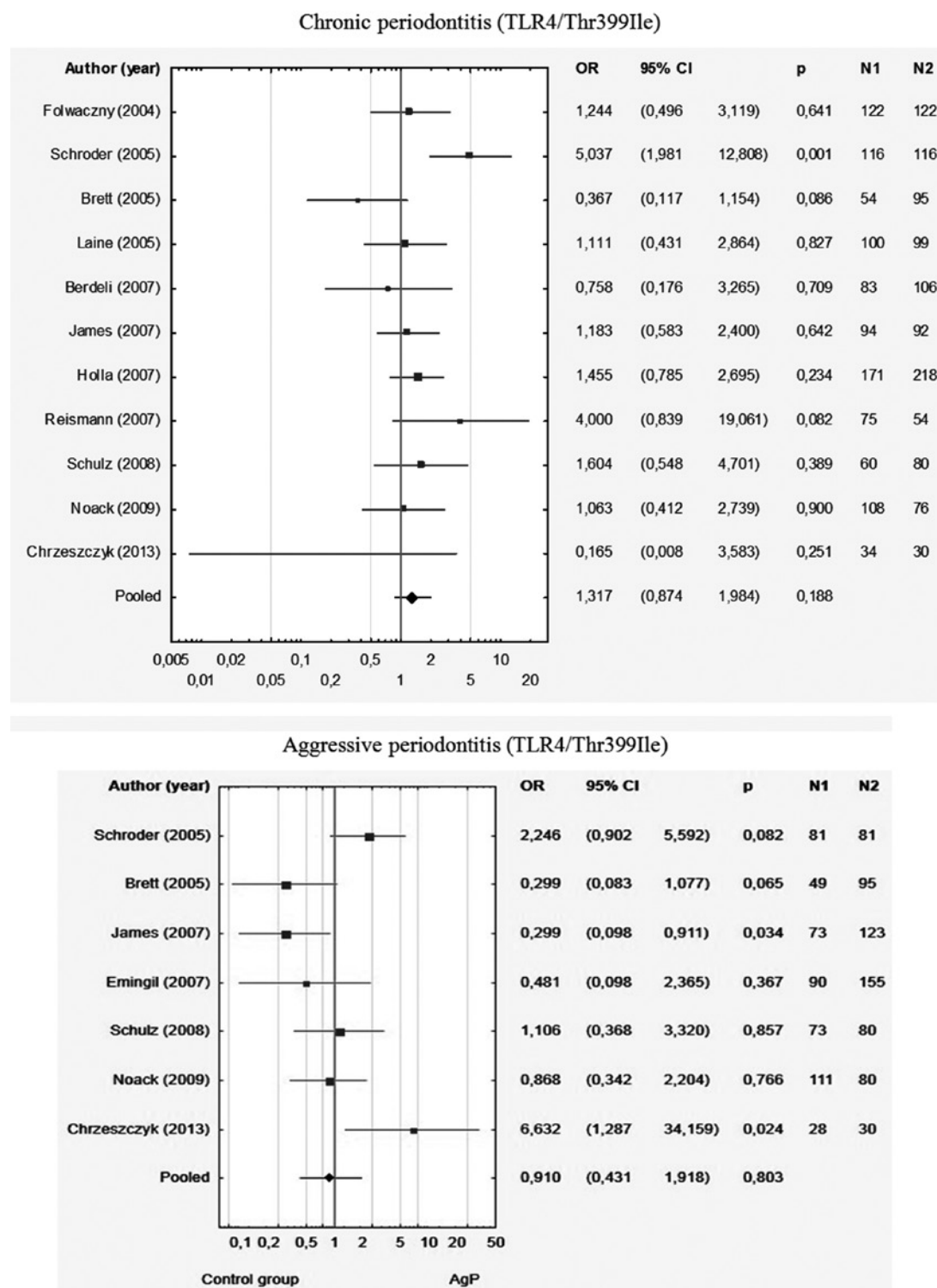


Fig. 7. Forest plot of published case-control studies of Thr399Ile TLR4 in CP and AgP

Discussion

Given non-representativeness of almost all papers on the prevalence of TLR4 polymorphisms in periodontitis in Caucasians (only one [25] included more than 150 people in the study group) and the discrepancies of their findings (3 studies showed a significant correlation, the other did not)

the use of meta-analysis to synthesize existing results is the most reasonable way.

Based on an analysis of 15 case-control studies (1,621 people in the study group and 1,755 in the control group for Asp299Gly and 1,522 and 1,461 respectively for the Thr399Ile) a significant correlation only between Asp299Gly allele occurrence and an increased risk of chronic periodontitis

Table 2. Comparison of past meta-analyses on TLR4 polymorphisms in periodontitis

Author (year)	To when	No. of studies	Periodontitis Asp299Gly Thr399Ile OR CI p OR CI p	CP Asp299Gly Thr399Ile OR CI p OR CI p	AgP Asp299Gly Thr399Ile OR CI p OR CI p
Ozturk et al. (2009)	June 2008	7 CP 4AgP	n.d.	1.43 1.31* (1.04–1.97) (0.72–2.39) 0.03 0.38	1.2* 0.55* (0.37–3.83) (0.15–2.01) 0.76 0.37
Song et al. (2013)	June 2012	11Per 5 CP 3AgP	0.98 1.03 (0.76–1.27) (0.75–1.41) 0.9 0.85	0.95 0.78 (0.68–1.33) (0.49–1.25) 0.78 0.3	1.6 1.32 (0.88–2.94) (0.6–2.9) 0.12 0.49
Ziakas et al. (2013)	3 March 2013	10 CP 5 AgP	n.d.	0.94 1.12 (0.75–1.18) (0.83–1.52) 0.68 0.74	1.04 0.79 (0.53–2.04) (0.42–1.65) 0.07 0.29
Own meta-analysis	31 Dez. 2013	13 CP 7 AgP	1.26 1.18* (0.95–1.69) (0.79–1.75) 0.11 0.42	1.35 1.31 (1.02–1.81) (0.87–1.98) 0.038 0.18	1.01 0.91* (0.58–2.07) (0.43–1.92) 0.77 0.8

*significant heterogeneity.

was shown. The frequency of wild type alleles in the CP in relation to the control group was 93.0 and 94.0 respectively. The distribution of genotypes Asp/Asp, Asp/Gly, Gly/Gly in CP was not in accordance with the Hardy-Weinberg rule ($p = 0.039$). The results of the three previously conducted meta-analyses on the association of TLR4 polymorphisms with periodontitis [14, 33, 34] are compared with present findings in Table 2. Our observations fully correspond to the first meta-analysis by Ozturk et al. [33]. Later studies carried out after June 2008 and included in our meta-analysis, only slightly influenced the pooled OR. Both of these meta-analyses are characterized by homogeneity of the studies included in the case of CP, and no grounds for stating publication bias. Most of the researches on TLR4 polymorphisms were carried out in Europe, where Asp299Gly and Thr399Ile are co-segregating SNPs. This haplotype is not associated with any changes of reactivity on bacterial LPS and the cytokine secretion profile and does not seem to show any association with susceptibility to infectious diseases [13]. Schröder et al. [19] showed in the case of Asp299Gly haplotype significantly increased susceptibility to chronic periodontitis. Differences with the results of other meta-analyses (correlations between Asp299Gly and CP) are due to the number and type of studies included in those papers.

Song et al. [34] for incomprehensible reasons, included only 5 studies describing the association of Asp299Gly and chronic periodontitis [18, 22, 23, 25, 31]. Some were considered as “unclassified periodontitis” [20, 21, 30], and others were overlooked [19, 26, 27, 30]. In the forest plot showing the general periodontitis, study by James et al. [22]

was quoted twice, and this has not happened in relation to research in CP and AgP by Brett et al. [20], Schulz et al. [28] and Noack et al. [30]. On the other hand Ziakas et al. [14] also did not include in their meta-analysis 3 studies [19, 26, 27], while the Brazilian study by Garlet et al. [35] in which 15 to 17% of people in three groups of subjects were African-American or Mulatto was used. Just how important is the uniformity of the race for the meta-analysis ensures stratification in own research of Turkish studies conducted in Izmir [23] and American conducted in Buffalo and Richmond [31] which showed an increase in the pooled OR from 1.35 to 1.51 (the decrease in the variance of the real effects T^2 to 0.003 and mean I^2 to 1.35%) while maintaining a significant deviation from the Hardy-Weinberg distribution.

Despite the slight differences in the rare allele Asp299Gly frequency between patients with CP and those with clinically healthy periodontium, most well-designed studies in Caucasians indicate the relationship of this polymorphism with the higher risk of this disease. Perhaps this mutation is responsible for the altered interactions between TLR4 present on immune cells (neutrophils, macrophages, dendritic cells, TregCD4+CD25+) and LPS. It may also influence the survival rate of periopathogenes in gingival pockets. However, this requires a more detailed analysis at the molecular level.

For aggressive periodontitis there is a consensus among all existing meta-analyses – both TLR4 polymorphisms do not significantly change the risk of this disease. In all meta-analyses, there was no publishing bias, which also reinforces this conclusion. In present meta-analysis which took into account most studies with clinical diagnosis of AgP

we showed no significant deviation in distribution of alleles Asp299Gly and Thr399Ile from the Hardy-Weinberg rule (p values were 0.224 and 0.222 respectively). Ozturk et al. [33] in the sensitivity analysis, after excluding the distorting homogeneity observation by Schröder et al. [19] showed a protective effect of Thr399Ile alleles against AgP development. These findings were not confirmed by later studies. The significant protective effect of this polymorphism was described by James et al. [22]. However, both British studies [20, 22] included in all the meta-analyses had control groups with unknown periodontal status and no information about other possible confounders (blood from blood banks). What is of interest is the fact that these two studies agree on the suggestion of a protective effect of Thr399Ile on susceptibility to AgP (for both OR was 0.3), and at the same time are completely contradictory with respect to the association of this polymorphism with CP (OR 0.3 and 2.78). Discrepancies in the results of correlations between both TLR4 polymorphisms and periodontitis are also related to differences in their co-segregation in different populations.

Interestingly, almost all individuals with TLR4 polymorphic variants in the examined groups were heterozygotes. Only James et al. [22] identified one homozygous person diagnosed with chronic periodontitis. It is possible that the phenotype of periodontitis is associated particularly with homozygous for the polymorphic variants of these receptors. The heterogeneity of the studies included in the meta-analysis describing the correlation of Thr399Ile TLR4 and AgP is probably due to the rarity of this type of periodontitis (prevalence is estimated at not more than 8% in populations under 35 years of age) and less numerous groups of patients. Only one study [29] recruited a fairly representative group of 111 people with AgP and an average age of 34 years.

Our meta-analysis has some significant limitations. Firstly, the interaction of genotype-phenotype-bacteria is affected by many factors (e.g., age, sex, smoking, socio-economic status). Reducing their impact can be achieved by including only the revised measure of the effect (the difficulty of obtaining such data) or pairing up groups in terms of their impact, which in relation to age, gender and smoking took place in only two studies [19, 25]. In extreme cases, when these factors were not taken into account, subjects with general diseases (e.g. diabetes) which can influence the course of periodontitis, were included in the study group [22, 26] or all information about control group's demographics were not available [20, 22]. Secondly, there were significant differences in the definition and classification of the study and control groups.

Most often, authors adopted their own, quite divergent definitions of periodontitis [18, 21–23, 27, 28, 31]. Sometimes the criteria of the American Academy of Periodontology [19, 20, 24, 25, 29, 30], the definition by Machtei et al. [26] or by Page and Ecke [32] were utilized. On the other hand, in some papers, measurements of alveolar bone loss on radiographs were used as a diagnostic criterion [18, 20, 21, 27–30]. Laine et al. [21] defined the test group as severe adult periodontitis based on the presence of at least 7 interproximal sites with more than 50% bone loss (in our analysis, it was considered chronic periodontitis). Differences in diagnostic criteria of periodontitis were the reason why we collectively evaluated the phenotype of periodontitis in our meta-analysis. Discrepancies were also observed in the case of the control groups – these were people without clinically visible signs of periodontitis [22, 23, 32], or with gingivitis [25, 26, 28]. Some authors believe that the best reference in SNP case-control studies should be chronic gingivitis, which presents the profile of resistant subject exposed to a microbial burden in periodontal environment [35]. Thirdly, it is also important to determine the effect of TLR4 polymorphisms on the clinical course of periodontitis. The effect of these SNPs on the variously defined severity of periodontitis or clinical parameters was assessed in ten studies [18, 23–30, 32]. Fourthly, the meta-analysis included small study groups (in two cases so small that in one test group and one control group polymorphic variant of TLR4 did not occur), affected by “small-study effects” – the results in small groups are different than in large ones. Conducted in all meta-analyses publication bias assessment (the Begg-Mazumdar test, the Egger test or the Harbord's test) did not confirm its existence, which reduces concerns about over-representation of small studies. Finally, haplotype-based methods are more sensitive methods of typing polymorphic susceptibility markers than SNP, particularly in the case of co-segregated alleles. Despite these limitations, the assessment of the overall effect which takes into account the variability of the results of individual studies is a useful method of evaluating the genetic markers of susceptibility and resistance to development and clinical course of periodontitis because of the practical impossibility to conduct a representative study in a single medical facility.

Several studies on the relationship of TLR4 polymorphisms and the risk of periodontitis were also carried out in Asia. Among the Mongoloid race Asp299Gly and Thr399Ile genotypes are virtually nonexistent. This was confirmed by Japanese [36] and Chinese [12] observations, which failed to show the presence of these alleles in any

patient with CP or AgP. However, in the Japanese population a significant association between polymorphic mutation in the 3' untranslated region of TLR4 gene (single nucleotide substitution G/C, -3725, rs11536889) and the risk of chronic periodontitis in both moderate and severe variants was reported [37]. Also, in the Indian population polymorphic allele Asp299Gly was not identified. In the group of 60 subjects diagnosed with chronic periodontitis there were three heterozygotes and one homozygote for Thr399Ile allele comparing to control group (60 participants with clinically healthy periodontium) where there was only one heterozygote for this allele [38]. These observations demonstrate the impossibility to include Asian studies in the meta-analysis on association between TLR4 SNPs occurring in Europe.

In summary, it can be stated that there is some evidence of an association between TLR4 gene mutation Asp299Gly and an increased risk of susceptibility to chronic periodontitis in Caucasians. This requires confirmation in molecular studies (evaluation of interactions between PAMPs and TLR4 present on immunological cells) and clinical trials (to assess the correlation between periodontal parameters and Asp299Gly haplotype). It seems that the search for a gene complex (SNPs) in conjunction with the occurrence of qualitative and quantitative spectrum of bacteria and viruses in periodontal pockets is the way to assess and maybe alter in the future the individual susceptibility to periodontitis [39]. Meta-analysis is surely the best means of identifying genetic markers and its importance cannot be underestimated.

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