SUMMARY
Background: Periodontitis is a chronic inflammatory disruption of the periodontal supportive tissues. There are the numerous evidences for his bacterial etiology. Though the occurrence of periodontal bacteria is considered to be the main cause of periodontitis, certain characteristics of the individual immune response may also have influence on the disease development and progression, and on the treatment outcomes. There are some reports that attempt to identify genetic factors associated with periodontitis including polymorphisms of interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) genes. We were interested from the distribution of several genotypes of the cytokines: interleukin-6 - (G-174C) and (G-597A), lymphotoxin-α (A+252G), and tumor necrosis factor-alpha (G-308A) in patients with chronic periodontitis.

Aim: To investigate the association of chronic periodontitis with certain gene polymorphisms of interleukin-6 (IL-6), Lymphotoxin-α, and tumor necrosis factor-alpha (TNF-α).

Material and methods: The study included 30 patients with moderate or severe chronic periodontitis, and 10 persons with healthy periodontium. Total genomic DNA was extracted from the buccal epithelial cells. TNF-A (G-308A), IL-6 (G-174C), IL-6 (G-597A) and LT-A (A+252G) genes polymorphisms were analyzed by Polymerase chain reaction (PCR).

Results: Outcomes showed a large variety in genotype’s distribution in the investigated groups. No important difference was observed in the distribution of IL-6, TNF-α and LT-α genotypes between chronic periodontitis patients and controls in this study be reason of the small studied group. However, a significant difference in the LT-α was observed – a prevalence of the genotype GG in patients with severe periodontitis. In relation with IL-6 (G-597A) and IL-6 (G-174C) genotyping - in both of them in patients with severe periodontitis was occurred most frequently the genotype GG. In patients with periodontitis the frequency of genotype GG of TNF-α (G-308A) was significantly increased.

Conclusion: The assessment IL-6 (G-597A) and IL-6 (G-174C), and TNF-α (G-308A) revealed that genotype GG was moderate associated with chronic periodontitis in Bulgarian individuals. As a result of these findings we may suppose that the G allele may play an important role in the development and progression of periodontal disease in this population. The frequency of LT-A (A252G) was significantly greater in severe periodontitis patients in this study.

Key words: gene polymorphism, interleukin-6, lymphotoxin-α, tumor necrosis factor-alpha, susceptibility, periodontitis.

INTRODUCTION:
At present it is known that pathogenic bacteria are the key factors for initiation of periodontal disease, but the host response and the severity of clinical expression are largely determined by genetic susceptibility and environmental factors. The first study of cytokine gene polymorphism was reported by Kornman [1] who found a significant association between severe adult periodontitis and composite genotype, namely, allele 2 of a single nucleotide polymorphism (SNP) of IL-1A+4845 and IL-1B+3954 located on chromosome 2q13. Following this, several studies have been conducted exploring the role of IL-1 gene polymorphism as a severity factor in periodontitis in various population and ethnic groups [2-4]. There are some reports that attempt to identify genetic factors associated with periodontitis including polymorphisms of interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) genes [2, 4-9].

In our study we were interested from the distribution of several genotypes of the cytokines: interleukin-6 - (G-174C) and (G-597A), lymphotoxin-α (A+252G), and tumor necrosis factor-alpha (G-308A) in patients with chronic periodontitis.

AIM:
To investigate the association of chronic periodontitis with certain gene polymorphisms of interleukin-6 (IL-6), Lymphotoxin-α, and tumor necrosis factor-alpha (TNF-α).

MATERIALS AND METHODS:
Forty patients - 30 patients with chronic periodontitis, and 10 - healthy subjects were included in the present study. Following the conventional periodontal examination clinical parameters - hygiene index, BoP (gingival bleeding index - bleeding on probing), PD (probing depth), CAL (clinical attachment loss) were recorded for the diagnosis. The subjects with periodontitis were devised into two groups of dif-
different disease severity based on CAL. Total genomic DNA was extracted from the buccal epithelial cells. TNF-α (G-308A), IL-6 (G-174C), IL-6 (G-597A) and LT-α (A+252G) genes polymorphisms were analyzed by Polymerase chain reaction (PCR). Then, PCR amplified products were directly digested with respective restriction enzymes (10U for HinIII and 20U for BseGI and NcoI) and the size controls and received bands were determined electrophoretically using 3% agarose gel (Thermo Scientific). The results obtained were analyzed statistically.

DNA extraction
Total genomic DNA was extracted from the buccal epithelial cells using GeneJet Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer’s instructions for buccal swabs and stored at -20°C in 70 µl of the Elution Buffer (Thermo Scientific).

Cytokine genotyping
TNF-α (G-308A), IL-6 (G-174C), IL-6 (G-597A) and LT-α (A+252G) genes polymorphisms were analyzed by Polymerase chain reaction (PCR) performed on the extracted DNA using Thermo Scientific Maxima Hot Start PCR Master Mix (2X), with the use of respective primer sets at the final concentration of 0.5 µM (Metabion International AG), that spanned the SNP sites, as described previously [2, 4, 5, 6] and PCR conditions as listed in Table 1.

Table 1.Primers, RCR conditions and restriction enzymes for different gene polymorphisms.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primers and PCR conditions</th>
<th>Restriction Enzyme</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
</table>
| TNF-A (G-308A)     | (F) 5’- AGG CAA TAG GTT TTG AGG GCC AT -3’  
(F) 5’- ACA CTC CCC ATC CTC CCG GCT-3’ | NeoI              | (4h, 37°C)        |
|                    | 95°C 4 min., (95°C 30 s; 60°C 30 s; 72°C 15 s) x 40, 72°C 5 min. |                   |                   |
| IL-6 (G-174C)      | (F) 5’- GGA GTC ACA CAC TCC ACC T -3’  
(R) 5’- CTG ATT GGA AAC CTT ATT AAG -3’ | HinIII=Hsp92II     | (4h, 37°C)        |
|                    | 95°C 4 min., (95°C 30 s; 57°C 30 s; 72°C 30 s) x 35, 72°C 5 min. |                   |                   |
| IL-6 (G-597A)      | (F) 5’- GAA GAC TTC AGG TGG TGG CAT T -3’  
(R) 5’- TCC TCG CGC ACA ACG GTC AGG GC -3’ | BseGI=FokI         | (4h, 55°C)        |
|                    | 95°C 4 min., (95°C 30 s; 65°C 30 s; 72°C 30 s) x 35, 72°C 5 min. |                   |                   |
| LT-A (A+252G)      | (F) 5’- TCC TCG CAG CTC CCT GGA TC -3’  
(R) 5’- GAG GAC ACG TGC AGG TGG TGT CAT T -3’ | NeoI              | (4h, 37°C)        |
|                    | 95°C 4 min., (95°C 30 s; 65°C 30 s; 72°C 30 s) x 35, 72°C 5 min. |                   |                   |

Then, PCR amplified products were directly digested with respective restriction enzymes (10U for HinIII and 20U for BseGI and NcoI). The size of both, the amplified DNA fragments (ND controls), and bands cleaved with restriction enzymes, was determined electrophoretically using 3% agarose gel (Thermo Scientific) stained with ethidium bromide (Sigma Aldrich) and Thermo Scientific GeneRuler Low Range DNA Ladder. Table 2 summarizes lengths of cleaved bands and respective polymorphisms.

Table 2. Different gene polymorphisms according to obtained DNA fragments.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>PCR amplicon / ND control size</th>
<th>Homozygous allele 1</th>
<th>Homozygous allele 2</th>
<th>Heterozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-A (G-308A)</td>
<td>117 bp</td>
<td>GG: 97 bp + 20bp</td>
<td>AA: 117 bp</td>
<td>GA: 117 bp + 97 bp + 20 bp</td>
</tr>
<tr>
<td>IL-6 (G-174C)</td>
<td>525 bp</td>
<td>GG: 327 bp +169 bp</td>
<td>CC: 327 bp +122bp +47 bp</td>
<td>GC: 327 bp + 169 bp +122 bp +47 bp</td>
</tr>
<tr>
<td>IL-6 (G-597A)</td>
<td>525 bp</td>
<td>GG: 525 bp</td>
<td>AA: 468 bp + 57 bp</td>
<td>GA: 525 bp + 468 bp + 57 bp</td>
</tr>
<tr>
<td>LT-A (A+252G)</td>
<td>368 bp</td>
<td>AA: 368 bp</td>
<td>GG: 235 bp + 133 bp</td>
<td>GA: 368 bp + 235 bp + 133 bp</td>
</tr>
</tbody>
</table>

RESULTS
The clinical parameters were analyzed among the selected patients (Table 3). Significant correlation between HI and PD, CAL and bone loss was observed. Similar results with a significant importance between PD and CAL, PD and Bone loss, and also between CAL and Bone loss was determined in this study (Table 4 and Fig. 1).
Table 3. Descriptive analysis.

<table>
<thead>
<tr>
<th>Description</th>
<th>Average values</th>
<th>Median</th>
<th>Standart deviation</th>
<th>Variety</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age Av</td>
<td>43,08</td>
<td>43,50</td>
<td>14,29</td>
<td>52,00</td>
<td>18,00</td>
<td>70,00</td>
<td>43,08</td>
</tr>
<tr>
<td>HI% Av</td>
<td>4,38</td>
<td>0,00</td>
<td>9,07</td>
<td>30,00</td>
<td>0,00</td>
<td>30,00</td>
<td>40</td>
</tr>
<tr>
<td>BoP Av</td>
<td>73,63</td>
<td>100,00</td>
<td>43,36</td>
<td>100,00</td>
<td>0,00</td>
<td>100,00</td>
<td>40</td>
</tr>
<tr>
<td>PD Av</td>
<td>3,82</td>
<td>3,65</td>
<td>0,76</td>
<td>3,30</td>
<td>2,80</td>
<td>6,10</td>
<td>30</td>
</tr>
<tr>
<td>CAL Av</td>
<td>4,45</td>
<td>4,20</td>
<td>1,13</td>
<td>4,30</td>
<td>3,10</td>
<td>7,40</td>
<td>30</td>
</tr>
<tr>
<td>Bone loss</td>
<td>3,12</td>
<td>2,65</td>
<td>1,40</td>
<td>5,50</td>
<td>1,50</td>
<td>7,00</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4. Correlation matrix (Rang – correlation analysis (Spearman))

<table>
<thead>
<tr>
<th></th>
<th>HI%</th>
<th>BoP</th>
<th>PD</th>
<th>Cal</th>
<th>BoneLoss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0,169</td>
<td>0,0213</td>
<td>0,111</td>
<td>0,148</td>
<td>-0,0257</td>
</tr>
<tr>
<td></td>
<td>0,368</td>
<td>0,91</td>
<td>0,556</td>
<td>0,433</td>
<td>0,892</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>HI%</td>
<td>-0,262</td>
<td>-0,467*</td>
<td>-0,432*</td>
<td>-0,505*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0,16</td>
<td>0,00953</td>
<td>0,0174</td>
<td>0,00464</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>BoP</td>
<td>0,208</td>
<td>0,124</td>
<td>0,268</td>
<td>0,197</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0,268</td>
<td>0,512</td>
<td>0,294</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>PD</td>
<td></td>
<td></td>
<td>0,815*</td>
<td>0,807*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Cal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0,778*</td>
</tr>
</tbody>
</table>

! *Significant correlations

Fig.1. Relationships between different parameters.
Results showed a large variety in genotype’s distribution in the investigated groups and are presented on Table 2 and Fig. 1-4.

Fig. 2. 3% agarose gel electrophoresis of RFLP with ethidium bromide staining of LT-A (A252G): Lanes 1, 6 and 16 - 100 bp ladder (L); Lanes 2, 5, 7, 11, 12 - heterozygous AG genotype with 3 fragments after restriction (368+235+133) length of the PCR product; ND – lane 10 – non digested control; Lanes 3, 8, 13, 14 – homozygous AA for allele 1 with one fragment (368); Lanes 4, 9, 15 – homozygous GG for allele 2 with two fragments (235+133).

![LT-A (A+252G) / Ncol](image1)

Fig. 3. 3% agarose gel electrophoresis of RFLP with ethidium bromide staining of IL-6 (G-597A): Lane 1 – ND control; Lanes 7 and 11 – ladder; Lanes 2, 3, 4, 6, 10, 14 – heterozygous GA genotype – two fragments (525+468); Lanes 9, 12, 13 – homozygous GG for allele 1 with one fragment (525); Lanes 5 and 8 - homozygous AA for allele 2 with two fragments (468+57).

![IL-6 (G-597A) / BseGI=FokI](image2)
Fig. 4. 3% agarose gel electrophoresis of RFLP with ethidium bromide staining of **IL-6 (G-174C)**: Lanes 2 and 7 – ND control; Lanes 1 and 13 – ladder; Lanes 5, 7, 8, 10, 11, 15 – heterozygous GC genotype – 4 fragments (327+169+122+47); Lanes 3, 4, 6, 10 – homozygous GG for allele 1 with 2 fragments (327+169); Lane 14 - homozygous CC for allele 2 with 3 fragments (327+122+47).

** Genotyping results: **
** IL-6 (G-174C) ** – in the control group was lack of genotype GG. In patients with severe periodontitis this genotype occurred most frequently.

** IL-6 (G-597A) ** – in the control group was lack of genotype GG. In patients with severe periodontitis this genotype occurred most frequently.

** LT-A (A252G) ** – in the control group was detected only genotype AG. In patients with chronic periodontitis were detected both AA and GG genotypes (in patients with severe periodontitis most frequently - genotype GG).

** TNF-A (G-308A) ** – in the control group most frequently was observed genotype GA (70% of cases). In patients with periodontitis the frequency of genotype GG was significantly increased. Only in one patient with severe periodontitis was observed genotype AA.

All results are presented on Table 5.
Assessment of the predictive value:

The regression analysis - Backward Elimination for prognosis verification was applied. The results obtained in this study showed that the periodontitis severity may be predicted base on LT-A (A252G) genotype. The total accuracy of conducted predictive model based on logistic regression was 70%.

Table 6. Classification table:

<table>
<thead>
<tr>
<th>Cases</th>
<th>Severity 1</th>
<th>Severity 2</th>
<th>Correctly predicted cases in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity 1</td>
<td>7</td>
<td>5</td>
<td>58.3%</td>
</tr>
<tr>
<td>Severity 2</td>
<td>4</td>
<td>14</td>
<td>77.8%</td>
</tr>
<tr>
<td>Total accuracy</td>
<td></td>
<td></td>
<td>70%</td>
</tr>
</tbody>
</table>

The combination of this genotype with the others of (IL-6 (G-174C), IL-6 (G-597A) and TNF-A (G-308A)) hasn’t exceeded the total precision of the model.

Following model has showed that the severe periodontitis have more frequently LT-A (A252G) AG genotype, whereas AA genotype was related with the moderate periodontitis.

DISCUSSION:

The current data show there are also investigations that attempt to identify genetic factors associated with periodontitis including polymorphisms of interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) genes. [10-16]

In this study our aim was to determine the distribution of TNF-A (G-308A), IL-6 (G-174C), IL-6 (G-597A) and LT-A (A+252G) genes polymorphisms in patients with chronic periodontitis. Interestingly the literature data have published mainly results obtained in several small and conflicting studies. [4, 5, 12, 14, 15, 17] Yoshie et al. reviewed the role of IL-6 polymorphism in periodontitis [12] and founded a correlation between IL-6 (G-174C) and chronic periodontitis susceptibility in Brazilian Caucasian population but not in Czech Caucasians. The studies of Brett et al., Babel and al. and Tervonen et al. showed correlation of IL-6 (G-174C) with susceptibility to chronic periodontitis [13, 14, 18]. Still more the investigation of Tervonen et al. was focused on polymorphism in the gene encoding IL-6, especially the IL-6-174 GG genotype, which is associated with prevalence of periodontal disease in patients with moderate and severe periodontitis. [18] This is in agreement with the results obtained in our study and this genotype was not presented in
control. In the present study was found an increased presence of IL-6 (G-174C) G/G genotype similarly to the results of Costa et al. In accordance to the other investigators we may conclude that G homozygous subjects (GG) were significantly more affected by periodontal disease than individuals who carry the C allele that was a great distribution in healthy individuals [5].

On the other hand, the Czech investigation suggested that the gene polymorphism IL-6 -572 may be regarded as a protective factor for the disease [17].

Regarding gene polymorphism of IL-6 -597 our results showed a prevalence of IL-6 (G-597A) GA genotype in healthy group and in moderate periodontitis group and thence may be related with the protective factors associated with lower susceptibility to chronic periodontitis.

There exist several investigation that support carriage of TNF-A (G-308A)*2 polymorphism with the severity of periodontitis in Caucasian population [10, 11]. Controversy, Fassman et al. demonstrated that TNF-A (G-308A) polymorphism have not association with chronic periodontitis in Czech population, whereas the LT-A (A252G) genotypes have statistically significant differences between periodontitis cases and healthy controls similarly to our study results. Therefore, according to the authors the LT-α gene may be considered an informative marker and it may be one of the protective genetic factors versus chronic periodontitis in our population [15].

As regard with the possible link between genetic variants and their expression, the polymorphisms in TNF-α were considered to be risk factors in periodontitis. There are several clinical studies that address to this suggestion. However, the results obtained from these studies are controversial [5, 10, 11, 14-16, 19-22].

As regards to TNF-A (G-308A) gene polymorphism in the present study G homozygous subjects were significantly more affected by periodontal disease than individuals who carry the A allele. These results also are in accordance with the results obtained in the study of Costa et al. [5].

From the review of Laine et al. it become clear that no gene polymorphism has, as yet, been definitely to be a risk factor for chronic periodontitis susceptibility. The explanation of this may be that small sample size studies, as in our study, are greatly underpowered since most associations have small odds ratio’s (1,1-1,5) and greatly contribute to the risk for false positive or negative outcomes [23].

In our study we investigated the IL-6 polymorphisms, at positions -597 and -174, TNF-α polymorphism at position -308, and LT-α polymorphism at position -252. No important difference was observed in the distribution of IL-6, TNF-α and LT-α genotypes between chronic periodontitis patients and controls in this study be reason of the small studied group. However, a significant difference in the LT-α was observed - a prevalence of the genotype GG in patients with severe periodontitis. In relation with IL-6 (G-597A) and IL-6(G-174C) genotyping - in both of them in patients with severe periodontitis was occurred most frequently the genotype GG. In patients with periodontitis the frequency of genotype GG of TNF-α (G-308A) was significantly increased. In addition, based on the results of this study and on the available literature, there are limited data to support associations between TNF-α gene polymorphism at position -308, IL-6 polymorphisms at positions -597 and -174, and LT-α polymorphism at position -252 and chronic periodontitis. On the other hand, the assessment IL-6 (G-597A) and IL-6(G-174C), and TNF-α (G-308A) revealed that genotype GG was moderate associated with chronic periodontitis in Bulgarian individuals. As a result of these findings we may suppose that the G allele may play an important role in the development and progression of periodontal disease in this population. The obtained results are in agreement with those of received in other investigations and authors and published in the specialized literature [5, 13, 14, 18, 22].

CONCLUSION:

The frequency of LT-α (A252G) was significantly greater in severe periodontitis patients in this study. However our investigation was limited on the number of subjects included. For that reason we may present our results only as a tendency or prevalence in determined genotypes of investigated genes without an important significance. At present, the results obtained in this study suggest that further studies are needed on gene polymorphisms of the major cytokines that are known to be involved in the protective-destructive immune response in periodontal diseases for definite conclusion.

Acknowledgements:

This work was supported by the Medical University of Sofia (grant No. 16/2014).

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Please cite this article as: Dossea-Panova V, Antoaneta Mlachkova A, Popova Ch, Kicheva M. Evaluation of interleukin-6, Lymphotoxin-α and TNF-α gene polymorphisms in chronic periodontitis. J of IMAB. 2015 Jul-Sep;21(3):868-875. DOI: http://dx.doi.org/10.5272/jimab.2015213.868

Received: 21/06/2015; Published online: 15/09/2015

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