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ABSTRACT

RESEARCH REGARDING THE ROLE OF OXIDATIVE STRESS IN THE ONSET OF AGGRESSIVE PERIODONTITIS - CORRELATIONS BETWEEN INFLAMMATORY CYTOKINES AND THE PERIODONTOPATHOGENIC BACTERIAL SPECIES INVOLVED

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KEYWORDS: aggressive periodontitis, periodontopathogenic bacterial species, IL-1 gene polymorphism, salivary biomarkers, salivary IL-1β, salivary 8-OHdG

INTRODUCTION

According to the WHO, periodontal disease is the main cause of tooth loss in adults, since the frequency of this disease in the world population is approximately 70%. According to most researchers and decision-making fora in the field of dentistry, both periodontal disease and dental caries are the major orodental problems of this century. In this context, the combined efforts of specialists in this field focus on discovering new information on the occurrence and evolution of this disease and identifying the best ways of diagnosis, treatment and prevention of periodontal disease occurrence.

Although periodontal disease has been known and treated over 5000 years, demonstrating the mechanism of occurrence of periodontal disease has changed over time, the phenomenon of disease being related to the rapid evolution of knowledge of microbiology and immunology, as well as the methods for estimating the clinical parameters (gingival sulcus and/or periodontal pockets) and through technological development. Thus, modern methods have identified as main pillars for the pathogenesis of periodontal disease the presence of periodontopathogenic bacteria and the intensity of the immune response, periodontal disease being currently listed as a „complex multifactorial etiology" disease.

In this thesis, we used a top molecular technique, namely polymerase chain reaction, DNA-strip technology. Through this technique, we intended to identify the involvement of five periodontopathogenic bacterial species with maximum potential in periodontal disease, namely: Aggregatibacter actinomycetemcomitans, Porphynomonas gingivalis, Tannerella forsythia, Treponema denticola and Prevotella intermedia.

A controversial factor involved in the occurrence of aggressive periodontal disease is the host genetic material, namely IL-1 gene polymorphism.

Along periodontopathogenic bacterial species and genetic predisposition, an important role in the early diagnosis and monitoring of periodontal disease is assigned to the markers of oxidative stress, since the role of reactive oxygen species in the altering of cellular DNA and all cellular homeostasis changes arising from this have been successfully demonstrated.

Beginning from these considerations, we had the idea for this thesis, in which we wanted to investigate the existence of possible correlation between proinflammatory cytokines, oxidative stress markers and periodontopathogenic bacterial species involved in the emergence of aggressive periodontal disease, as well as trying to achieve an early diagnosis and monitoring scheme of the disease in young people.

CHAPTER 1. HIGHLIGHTS OF AGGRESSIVE PERIODONTITIS IN THE PRESENT

Aggressive periodontal disease has the following characteristics: the loss of the tooth attachment structures accompanied by progressive destruction of the alveolar bone, the bacterial load which is not correlated with the severity of the disease, familial aggregation, frequently *Actinobacilus actinomycetemcomitans* infection is present, the abnormal function of phagocytes and an amplified response from macrophages with the production of prostaglandins [3, 10].

The World Health Organization declared in 2012 an incidence of periodontal disease of 15-20% [11] and suggests as prevention strategies the implementation of programs designed to interrupt tobacco use and to implement good practices for oro-dental health, such as brushing and flossing [12].

Socransky et al. [23] specify the organization of periodontopathogenic bacterial species present in periodontal areas in a „bacterial complex”, which acts through successive associations until specific lesions of periodontal disease appear [24].

CHAPTER 2. CYTOKINES

All body cells, including those that are part of the immune system, realize information exchanges through mediators, forming the superfamilies of cytokines or chemokines, with a major role in the body's defense system, being involved mainly in multiple pathological phenomena [33] .

In the inflammatory response, as part of the immune response, proinflammatory cytokines, which have a downstream activation sequence (the sequence TNF-α, IL-1 beta, IL-6, IL-8, IFN-γ), counteract the protective effect of anti-inflammatory cytokine [38].

Genes which encode IL-1 include the IL-1A, IL-1B and IL-1RN, which are located in the same chromosomal region 2q13 (chromosome 2, the long arm, region 1, band 3).

CHAPTER 3. OXIDATIVE STRESS MARKERS IN PERIODONTAL DISEASE

Biomarkers are molecules produced in the healthy individual’s body or in patients who have a disease and are used in clinical status monitoring, in identification of the disease onset or for the response obtained after treatment [59]. Saliva can be used to monitor the overall health status and to identify the onset of certain diseases [63].

Hydrogen peroxide can pass through the nuclear membrane and cause changes in the DNA, including nucleotide oxidation [77]. 8-hydroxy-2-deoxyguanosine (8-OHdG) is an oxidized nucleotide that is expelled into the saliva, numerous studies showing that the amounts of this biomarker are useful in quantifying the oxidative stress from the periodontal disease [65, 66, 70, 77].

CHAPTER 4. GENETIC KNOWLEDGE IN THE DIAGNOSIS OF PERIODONTAL DISEASE

Some of the first genetic researches in periodontal disease were those made by Kornman KS [88] in 1997 who studied interleukin-1

genotype as a severity factor of this disease. If the studies made until that moment had shown the relationship between periodontopathogenic bacterial species, smoking and periodontal disease progression, the author emphasizes that the IL-1 genotype may be a useful predictor of this disease in the case of a group of non-smokers. He believes that although periodontal disease can be diagnosed clinically, for the first time it is possible for doctors to use a genetic marker to identify individuals susceptible to periodontal disease, with the exception of the degree of bacterial load.

We conclude that aggressive periodontal disease is the phenotypic expression of environmental factors (smoking status, eating habits, stress), of genetic profiling (polymorphisms) and of biological interactions (gene-gene or gene-environment) [101].

CHAPTER 5. WORKING HYPOTHESIS. GENERAL OBJECTIVES.

5.1 PURPOSE OF THE STUDY

The aim of the study is the fundamental research through genetics, biochemistry and microbiology studies in aggressive periodontal disease.

5.2 OBJECTIVES OF THE STUDY

The overall objective of this study is to investigate the existence of a possible correlation between proinflammatory cytokines, oxidative stress markers and periodontopathogenic bacterial species involved in the emergence of aggressive periodontal disease, in the hope of achieving an early diagnosis and monitoring scheme of this disease in young people.

CHAPTER 6. GENERAL METHODOLOGY

6.1 STUDY GROUP STRUCTURE

Population: The study group consists of people with ages between 35 and 44 from Constanta, Romania.

Sample group: To establish the representative sample for this age group, the multistage stratified sampling method was used, carried out on three levels: the living environment level (rural, urban), the locality level (proportional random selection) and the family medicine unit level (proportional random selection). Studied samples were then extracted through systematic selection by statistical step, using as a sampling frame the complete lists of patients from family doctors in the units selected for the study. The sample which was initially necessary, calculated for a confidence level of 95%, with a sampling error of 6% and an estimated level of smoking and periodontal disease of 50% in the studied population, consisted of 379 subjects with ages between 35 and 44. [102, 103, 104].

6.2 DISTRIBUTION OF SAMPLE GROUP

The response rate was 77.30% (86 subjects refused participation in the study). After the exclusion of 7 subjects from the study (tobacco sellers), the final sample consisted of 286 subjects (6% sampling error, 95% confidence level) with an average age of 484.01 ± 41.80 months, 38.1% men (n = 109) and 61.9% women (n = 177) [102, 103, 104].

6.3 CLINICAL EXAMINATION

Clinical examination of the oral cavity was performed by inspection, palpation and percussion, with emphasis on periodontal examination. We kept a record of the CPITN INDEX (Community Periodontal Index of Treatment Need).

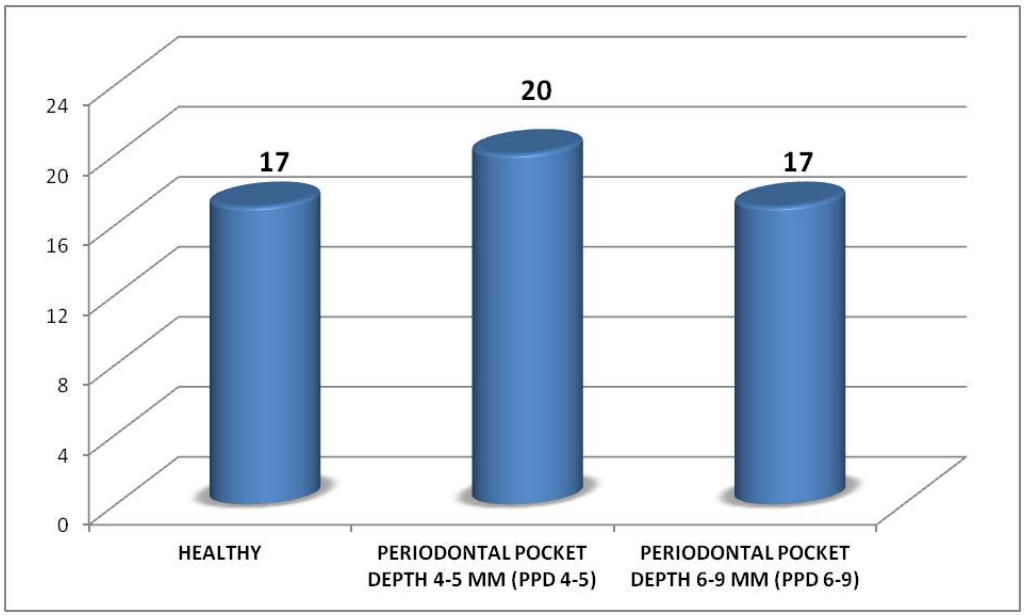
The dstribution of the maximum CPITN scores showed that only 8% (n = 23) of the subjects had a healthy periodontium, 42.7% had plaque and 46.5% presented periodontal pockets (n = 133).

In the present study the following subjects were included:

• all non-smoking healthy subjects (17 subjects of the 23 subjects with a 0 CPITN INDEX);

• all non-smoking subjects who had aggressive periodontitis (37 subjects of the 133 subjects with various stages of periodontal disease and periodontal pockets exceeding 3 mm).

In accordance with the specialized literature, we have divided the study group (Figure 1) in: the control group (healthy), in which we included subjects with PPD from 0 to 3 mm, the group of patients with average periodontal pocket depth between 4 and 5 mm (PPD 4-5), and the group of patients with periodontal pocket depth between 6 and 9 mm (PPD 6-9) according to Silvestre FJ et al. [105].



Graphic 1. Distribution of subjects in study groups

We obtained the approval of the Ethics Committee from the Ovidius University from Constanta in order to comply with the ethical principles of medical research involving human subjects. The subjects were informed about the purpose of the investigations and they participated in the study on the basis of an informed consent which they were asked to sign.

6.4 SAMPLE COLLECTION AND TRANSPORT

The sample collection was performed in the same session as the clinical examination but immediately prior to it, in order to avoid possible contamination with blood of oral fluid samples. The collection of saliva was done by different techniques depending on the laboratory methods used and it will be described in each study.

6.5 GENETIC METHODS

The genetics method used to identify periodontopathogenic bacterial species and IL-1 genotype was Polymerase chain reaction - Strip-DNA technique.

6.6 IMMUNOLOGICAL METHODS

As immunological method, we used the ELISA method. [109, 110]

6.7 STATISTICAL ANALYSIS

The results were statistically analyzed by using the program IBM SPSS STATISTICS 20.

CHAPTER 7. STUDY I - PERIODONTOPATHOGENIC BACTERIAL SPECIES AND AGGRESSIVE PERIODONTITIS

7.1 INTRODUCTION

The purpose of this research is to highlight the possible relationship between periodontopathogenic bacterial species and their possible involvement in the onset of aggressive periodontal disease by using a top identification method, i.e. the polymerase chain reaction - DNA strip.

7.2 WORKING HYPOTHESIS

The main objective of this study is to investigate the correlations between periodontopathogenic bacterial species involved in the

emergence of aggressive periodontal disease, in order to achieve an early diagnosis and monitoring scheme of this disease in young people.

7.3 MATERIAL AND METHOD

7.3.1 Collection of samples

We collected samples from five periodontal pockets of each subject. We used a collection kit manufactured by Hain Lifescience.

7.3.2 Assay Protocol

MicroIDent® test is based on the DNAstrip technology and it allows the molecular genetic identification of five combined periodontopathogenic bacterial species, namely: *Aggregatibacter actinomycetemcomitans, Porphynomonas gingivalis, Prevotella intermedia, Tannerella forsythia* and *Treponema denticola*. [107]

7.4 RESULTS

According to the classification given by Socransky and supported by the manufacturer of the analysis material, we have grouped the periodontopathogenic bacterial species in three classes: The Aa complex (containing only bacteria from *Aggregatibacter actinomycetemcomitans* - *Aa* group), the red complex (comprising bacteria from *Porphynomonas gingivalis* - *Pg*, *Tannerella forsythia* - *Tf, Treponema denticola – Td* classes) and the orange complex (which includes *Prevotella intermedia – Pi*).

We chose to evaluate the statistical significance of periodontopathogenic bacterial species separately, from the highest bacterial species in terms of pathogenicity (*Aggregatibacter actinomycetemcomitans*), the red complex bacteria species and *Pi* considered separately, then combinations of bacterial species *Aa* and red complex, bacterial species of the red complex coupled with *Pi*, *Aa* and *Pi* and finally, a combination of all bacterial species. We made this choice based on data from the speciality literature and a statistical

estimation regarding all bacterial species considered in the study according to the molecular identification method used in our research.

For all the evaluated scenarios, we noticed that there is a statistically significant difference regarding the presence of these bacterial species between the healthy group and that with aggressive periodontal disease, both among healthy subjects with PPD 4-5, $p < 0.05$, and between healthy subjects and PPD 6-9, $p < 0.05$.

It has been noted that between the two groups of patients there was not any statistically significant difference regarding the presence of bacterial species, $p > 0.05$, this situation confirming once again the position of these periodontopathogenic bacterial species in patients with aggressive periodontal disease, regardless of the depth of the periodontal pocket.

7.5 DISCUSSIONS

As most studies support, aggressive periodontal disease is a multifactorial disease having as an etiological factor the presence of bacterial microflora. The most common bacterial species studied and associated with the presence of aggressive periodontal disease are *A. actinomycetemcomitans* and *P. gingivalis*, and the list is completed by *P. intermedia, T. forsythia* and *T. denticola* [117].

Our results are consistent with the majority of studies that indicate that, in fact, *A. actinomycetemcomitans* is associated with aggressive periodontal disease [118, 119].

Our results show that the association between *A. actinomycetemcomitans* and *P. intermedia* is present in the patient groups evaluated in the study.

In a study conducted in Japan by using PCR, the authors showed that *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* were found in high numbers in periodontal pockets in patients enrolled in the study and furthermore, that there are statistically significant differences between the presence of these bacterial species in patients

with aggressive periodontal disease than in those with the chronic form [121]. The results are similar to those obtained by us, the association between *A. actinomycetemcomitans* and red-complex bacteria species being common in our patients.

A very recent study (2014, Wang X. et al.) conducted on a population group from China, shows that the presence of *A. actinomycetemcomitans* may be correlated with aggressive periodontal disease [123]; authors identify the presence of these bacterial species both in patients with small pockets and in those with deep pockets, as indicated by our study results.

We can say that, at the moment, the opinion of most experts, about the involvement of periodontopathogenic bacterial species in etiopathogenesis of aggressive periodontal disease is divided. In this context, Kononen E. and Muller HP show in their study that, although in recent decades researchers have given importance to the association between *A. actinomycetemcomitans* and aggressive periodontal disease, authors consider these bacterial species as a minor component of the bacterial flora from the oral cavity and only in some individuals it serves as an opportunistic pathogen. The authors consider as pathogens in the periodontal aggressive disease the bacterial species from the red and orange complexes. Also, the same authors reveal that studies are conducted on different populations in terms of geographical location and ethnicity, something that should be considered when making comparisons between different outcomes [125].

7.6 PRELIMINARY CONCLUSIONS

1. ***Our study demonstrates that there is a close correlation between the presence of periodontopathogenic the bacterial species identified and the clinical status expressed by periodontal pocket depth, elements that support the involvement of bacteria in the etiology of the aggressive periodontal disease;***

2. ***The highest correlation was found between periodontal the pocket depth and the periodontopathogenic bacterial species A.actinomycetemcomitans, followed by the associations between A.actinomycetemcomitans andP.intermedia or the red complex;***
3. ***The weakest correlation is between the periodontal pocket depth and the presence of periodontopathogenic bacterial species within the red complex and/or P. intermedia;***
4. ***Our study shows that there are no statistically significant differences between the presence of the periodontopathogenic bacterial species and the size of the periodontal pocket depth.***

CHAPTER 8. STUDY II - INTERLEUKIN 1 AND AGGRESSIVE PERIODONTAL DISEASE

8.1 INTRODUCTION

The purpose of this phase of the research is to highlight the possible relationship between the identification of the genetic profile linked to IL-1 gene polymorphisms and salivary IL-1β as oxidative stress biomarker in patients with aggressive periodontal disease, by using a top identification method, polymerase chain reaction – DNA-strip, respectively the ELISA technique.

8.2 WORKING HYPOTHESIS

The general objective of this study is to investigate the correlations between the genetic profile linked to IL-1gene polymorphism, salivary IL-1β proinflammatory cytokine and the onset of aggressive periodontal disease, in order to achieve an early diagnosis and monitoring scheme of this disease in young people.

8.3 MATERIAL AND METHOD

8.3.1 Collecting samples for genetic test

We collected saliva samples from each subject. We used a collection kit manufactured by Hain Lifescience.

8.3.2 Collecting samples for salivary IL-1β

For ELISA, saliva samples were collected by standard method (passive saliva collection in sterile containers). [109]

8.3.3 Analysis protocol for molecular biology

The IL-1® genotype test is based on the DNA-strip technology and it allows combined molecular genetic identification of IL-1A -889, IL-1B +3953and IL-1RN +2018 polymorphism of interleukin 1 gene locus. [108]

8.3.4 Protocol analysis of ELISA for salivary IL-1β (Promokine)

For the determination of the IL-1β biomarker we used the ELISA method, the sandwich technique, according to the manufacturer's instructions (Promokine, USA).

8.4 RESULTS

It is essential to point out that most subjects from our study have heterozygous profile (CT) and belong to the group of patients with aggressive periodontal disease. In the healthy group, we identifyed a few subjects with homozygous TT genotype for IL-1 genes.

If we consider the IL1A genotype frequency in the studied groups, we observe that there is a statistically significant difference regarding the presence of the CT genotype between healthy and aggressive periodontal disease groups, $p = 0.0237$. For the presence of the TT genotype between the healthy and aggressive periodontal disease groups, we obtained a highly significant statistical difference, $p = 0.0006$. However, no significant statistical difference was found between the presence of the CC genotype in the healthy and aggressive periodontal disease groups, $p = 0.7349$.

Regarding the frequency of the IL1B genotypes in the study groups, we have noticed that there is a highly statistically significant difference both between the presence of the CT genotype identified in

the healthy and aggressive periodontal disease groups, p = 0.0015, and also between the presence of the TT genotype in the two groups of evaluated subjects, p = 0.0001. Also, we did not obtain a statistically significant difference between the presence of the CC genotype in the healthy and aggressive periodontal disease groups, p = 0.4944.

For the IL-1B gene we have also obtained high statistically significant differences (p = 0.000) between the values of IL-1β and all the three genotypes identified by PCR.

8.5 DISCUSSIONS

In the medical literature there are studies that have identified correlations between the genetic modification of the IL-1 gene and the existence of aggressive periodontal disease [126, 127, 128, 129]. There are few studies in the world which have managed to present a correlation between the genetic defect, phenotypic quantification of IL-1 and disease status. In Romania, such studies are absent, which allows us to affirm that our results are a novelty.

The current medical literature presents studies that support the existence of an association between IL-1 gene polymorphism and aggressive periodontal disease. Thus, Safonov A.V. et al. [132] shows that the IL-1B genetic defect affects its phenotypic expression and in association with IL-6 polymorphism can lead to an increased severity of the disease; in this context, it was noted that in this association *A. actinomycetemcomitans* with *P. gingivalis* have been identified, outlining a new paradigm linked to the correlation between the polymorphism of these genes, the phenotypic expression and the possible potentiation of the colonization with periodontopathogenic bacterial species in the presence of genetic defects.

In the last 10 years, several studies have shown that the severity of periodontal disease is closely related to IL-1 gene polymorphism and local inflammatory mediators, among which IL-1 beta plays an important role [134]; Thus, Guzeldemir et al. [135] identified a highly

statistically significant association between IL-1 gene polymorphism and aggressive periodontal disease in study group from Turkey. Also, according to Quappe et al. [136] in the case of the Chilean population, IL-1 gene polymorphisms have been identified in 25% of patients with early-onset periodontal disease. In another study conducted on a sample population of Denmark, Havemose-Poulsen et al. [137] have identified an association between IL-1 genotypes and aggressive periodontal disease. Similar results were reported by Parkhill [138] who has discovered an association between aggressive periodontitis and the prevalence of IL-1 gene polymorphism in a population group from UK.

Our results are very similar to those of the authors mentioned above, with a highly statistically significant difference between the presence of IL-1 gene polymorphism and aggressive periodontal disease (IL-1B TTgenetic profile).

The correlation between IL-1B gene polymorphism and gene expression reflected by the amount of IL-1beta synthesized, remains a controversial topic, expecting that, in the near future, the expansion of the studies to larger groups of subjects and genetically diverse populations to lead to the elucidation of this subject. In this context, in the current literature the results of studies that show no correlation between IL-1 gene polymorphism and aggressive periodontal disease are presented; thus, the results were realized on different genetic groups - Caucasian, African, East Asia [97, 99, 139, 140, 141, 142, 143, 144].

Most authors listed above conclude that it is possible to have more than one genetic defects and that the disease may be caused by alterations of genetic material from multiple genes [97, 143, 145].

8.6 PRELIMINARY CONCLUSIONS

1. ***Our study shows that there are statistically significant differences between the genetic profile of IL-1 in patients with aggressive periodontal disease compared to healthy group;***
2. ***Regarding the association between genetic profile and the disease phenomenon, our results demonstrate that there is a correlation (very high for IL-1B and high in IL-1A) between genetic polymorphisms and clinical status;***
3. ***The highest statistical significance was achieved for the presence of the TT genotype in all IL-1 genes studied, followed by the CT genotype;***
4. ***There is no statistically significant difference between the presence of the CC genotype in healthy subjects and patients with aggressive periodontal disease for any of the IL-1 genes evaluated;***
5. ***There is a correlation between the genetic profile of IL-1B and their phenotypic expression, respectively the amount of IL-1beta measured in saliva.***

CHAPTER 9. STUDY III - OXIDATIVE STRESS BIOMARKERS AND AGGRESSIVE PERIODONTITIS

9.1 INTRODUCTION

The purpose of this phase of the research is to highlight the possible relationship between disease status (expressed by periodontal pocket depth), genetic profile (IL-1 gene polymorphisms) and oxidative stress biomarkers (salivaryIL-1β and 8-OHdG).

9.2 WORKING HYPOTHESIS

The main objective of this study is to investigate the correlations between disease status, proinflammatory cytokines and oxidative stress markers involved in the onset of aggressive periodontal disease,

for obtaining a scheme of early diagnosis and monitoring of this disease in young people.

9.3 MATERIAL AND METHOD

We collected saliva samples from each subject. For genetic tests and for the quantitative determination of salivary IL-1β, saliva harvesting methods were described in the above mentioned studies.

For the 8-OHdG the obtained samples were centrifuged at 8000 rpm for 10 minutes. [110]

9.3.1 Assay Protocol

For the genetic tests and salivary IL-1β, analysis protocols we presented in the previous chapters. In this study, I will present the ELISA technique for determining salivary 8-OHdG.

9.3.2 ELISA principle for salivary 8-OHdG

For the determination of the 8-OHdG biomarker we used the ELISA technique, the competitive method, as described by the manufacturer (Cayman Chemical, USA).

9.4 RESULTS

9.4.1 The results obtained for IL-1A (-889), the diseasestatus and phenotypic parameters

There is a highly significant difference ($p < 0.001$) between the mean values of the amount of salivary IL-1beta associated to the clinical status and IL-1 Agenotype.

By analyzing the results, we can see that the influence of the independent variables (clinical status and IL-1Agenotype) on the dependent variable (salivary IL-1beta) introduced the Two Way ANOVA model is highly significant statistically ($p < 0.001$).

Also note that there is a strong correlation between clinical status, IL-1A genotypes and the amount of salivary 8-OHdG. Analyzing the results, we noted that the influence of the parameters

entered in the Two Way ANOVA model is highly significant statistically ($p < 0.001$).

9.4.2 The results obtained for IL-1B (3954), the disease status and phenotypic parameters

The results indicate that there is a strong correlation between the clinical status, IL-1B genotypes and the amount of salivary IL-1beta. From the analysis of our results, it is noted that the influence of the factors introduced into the Two Way Anova model is highly significant statistically ($p < 0.001$).

There is a strong correlation between clinical status, IL-1B genotypes and the amount of salivary 8-OHdG. Also, for 8-OHdG, we can say that there is a highly statistically significant influence of the factors introduced in the Two Way Anova model($p < 0.001$).

9.5 DISCUSSIONS

Our study is an absolute novelty in Romania, because an assessment of genetic changes in the IL-1 gene in parallel with the presence of bacteria and oxidative stress in the context of aggressive periodontal disease has not yet been conducted.

In the medical literature there are many studies that prove the existence of an association between salivary levels of 8-OHdG and the presence of aggressive periodontal disease. Sezer et al. [65] claims in his study that there is a highly statistically significant correlation between the values of salivary 8-OHdG and clinical indexes. Also, Canakci et al. [64, 76] have shown in several studies conducted on patients with aggressive periodontal disease that the salivary level of 8-OHdG is a biomarker that is identified early in the event of distortion of mitochondrial DNA from the gingival tissue due to oxidative processes.

Takanori et al. [146] also indicated in his study that the level of salivary 8-OHdG is strongly correlated with periodontal pocket depth; Moreover, the author states that this biomarker can be successfully

used in the diagnosis and monitoring of patients with periodontal disease.

In our previous studies [67, 68, 69] we have shown that salivary 8-OHdG is a sensitive biomarker that becames positive even before clinical signs of periodontal disease, which proposes it as a very useful instrument in the daily practice of dentists in order to achieve early diagnosis of periodontal disease, being a potential screening tool of this disease.

Moreover, there are numerous studies that successfully demonstrate the utility of this salivary biomarker and they propose it as a tool for monitoring the effectiveness of periodontal disease treatment [66, 68, 69, 146].

We have demonstrated that the presence of periodontopathogenic bacterial species, IL-1 genotype-phenotype and the amount of salivary 8-OHdG are in strong correlation with the oro-dental clinical status and that there are no studies in the medical literature correlating the parameters mentioned above.

9.6 PRELIMINARY CONCLUSIONS

1. ***Our study demonstrates that there is a strong link between genetic profile and aggressive periodontal disease;***
2. ***There is an association between genetic polymorphisms and the evaluation of the inflammatory process and oxidative stress levels through the values of salivary IL-1beta and 8-OHdG determined in aggressive periodontal disease.***

CHAPTER 10. SENSITIVITY AND SPECIFICITY EVALUATION OF THE USED TESTS

To demonstrate the value of paraclinical tests used in our research and creating a hierarchy, we performed a statistical analysis on their sensitivity and specificity.

Given that in our days the PCR technique is appreciated by specialists as the most sensitive and most specific way to identify and quantify a parameter, we decided to analyze the sensitivity and specificity of the ELISA method used for quantification of salivary IL-1β and 8-OHdG. We conducted this evaluation by using the ROC curve, the curve that also identifies the cut-off value which distinguishes between the normal and pathological values.

The estimated value (cut-off value) for the IL-1β variable that can distinguish between the two groups (healthy and diseased), in terms of maximum sensitivity (Se) and specificity (Sp), is 30.001 pg/mL.

The area under the ROC curve plotted for the variable IL1beta (A = 0.943) differs significantly from the value 0.5 (1/2 of the square area). The calculated probability associated with this value is $p = 0.0001 < \alpha = 0.05$. In these circumstances, it can be said that the specified variable has the ability to distinguish between the two groups - healthy and patients with aggressive periodontal disease.

The estimated value (cut-off value) for the 8-OHdG variable that can distinguish between the healthy and the aggressive periodontal disease groups, in terms of maximum sensitivity (Se) and specificity (Sp), is 1.43 ng/mL.

Also, for 8-OHdG, the area under the ROC curve (A = 0.982) differs significantly from the 0.5 value (1/2 of the square area). The calculated probability associated with that value is $p = 0.0001 < \alpha = 0.05$. Thus we can say that this variable has the ability to distinguish between healthy patients and those with aggressive periodontal disease.

Next, we compared the ROC curves to determine which of the two variables can accurately distinguish between the two groups, or in other words, which of the two tests is more sensitive.

Given the test results, the difference between the areas is 0.039 at a statistical significant level of p = 0.231> 0.05, so we conclude that the two tests are almost equal in terms of sensitivity.

CHAPTER 11. STATISTICAL ESTIMATION OF DISEASE PROGRESSION RISK

Relative risk (RR), i.e. the ratio between the proportions of cases that produced a positive event (*Aa* = PRESENT) in the two groups of patients with aggressive periodontal disease vs. healthy is 10.56, a statistically significant value (95% confidence interval CI = 1.55 to 71.93).

Given that bacterial species are also identified in healthy subjects, we considered it useful to calculate the relative risk of disease progression in terms of the presence of these bacterial species. Thus, identifying *Aa* bacterial species present in patients with aggressive periodontal disease leads to a progression of 10.56 times higher than in healthy periodontal subjects who had this bacterial species identified.

Similarly, the association between the presence of *Aa* species with salivary IL-1β leads to a disease progression of 12.48 times higher in patients with aggressive periodontal disease, while the combination of salivary 8-OHdG and *Aa* presence leads to a disease progression of 4.63 higher in patients with aggressive periodontal disease.

If elevated amounts of salivary IL-1β are associated with 8-OHdG, there is a progression level of 3.47 times higher in patients with aggressive periodontal disease.

For the association of genetic variants of IL-1B and salivary IL-1β, it was noted that the presence of the CT genotype leads to a progression of 4.72 times higher in patients with aggressive periodontal disease and the presence of the TT genotype leads to a 5.23 times higher progression of the disease.

The association between periodontal pocket depth and the presence of *Aa* leads to a progression of 2.48 times higher in patients with aggressive periodontal disease; also the combination of PPD with salivary IL-1β leads to a 6.09 times higher progression of the disease, while the combination of PPD with salivary 8-OHdG leads to disease progression of 17.41 times higher in patients with aggressive periodontal disease.

CHAPTER 12. ORIGINALITY AND INNOVATIVE CONTRIBUTIONS OF THE THESIS

The originality of this thesis is the parallel evaluation of some oxidative stress markers of periodontal disease estimation, given that, in the medical literature, they are estimated separately.

The idea of this parallelism arose from the current state of knowledge related to the etiopathogenesis of periodontal disease, more and more factors directly and indirectly involved in the development of this disease are discovered.

We also consider that the originality of the thesis lies in how the research correlated the oxidative stress study with proinflammatory cytokines and periodontopathogenic bacterial species in parallel with genetic modification, respectively the polymorphism of some genes.

The accuracy of the results obtained by high performance techniques - PCR - support the implementation of genetic tests in the proces of identifying subjects exhibiting IL-1 gene polymorphism and, in consequence, an increased risk for periodontal disease.

The results of the thesis bring innovative contributions to updating the knowledge of diagnosis, monitoring and prognosis of periodontal disease. At the same time, we believe that an innovative contribution of the thesis consists in the possibility of using salivary 8-OHdG biomarker in the periodontal disease screening.

Our results support the possibility of using as biomarker the *A. actinomycetemcomitans*species, given that this species has been

identified in healthy subjects which, in time, will require a close monitoring in order to prevent the onset of periodontal disease.
In the end, we believe that all these arguments support the contribution of the thesis results to the optimization of the diagnostic scheme for periodontal disease.

CAP. 13 FINAL CONCLUSIONS

- The results we obtained regarding the assessment of all studied parameters, in relation to the normal and pathological states of the periodontium, show that each of them is relevant in the diagnosis of the aggressive periodontal disease.
- Genetic studies on the identification of periodontopathogenic bacterial species demonstrate that A. actinomycetemcomitans and the combination of A. actinomycetemcomitans with P. intermedia were the most frequently identified microbial species that produce the aggressive periodontal disease.
- Studies on the genetic profile related to IL-1 genes show that their polymorphism is closely linked to the aggressive periodontal disease.
- There is a high correlation between the genetic profile of IL-1B, respectively polymorphisms of these genes and phenotypic expression, i.e. the amount of IL-1beta quantified in saliva.
- IL-1 gene polymorphism is correlated with the clinical status and the amount of 8-OHdG and salivary IL-1 beta quantified.
- Following these results, we can estimate that clinically oro-dental healthy subjects who present IL-1B gene polymorphism require special monitoring in order to prevent the occurrence of the periodontal disease.
- The two parameters evaluated quantitatively (salivary IL-1beta and 8-OHdG) by ELISA reflect, with a high level of sensitivity and specificity, their involvement in aggressive periodontal disease, results that support the use of these biomarkers in the diagnosis and monitoring of the periodontal disease.
- The statistical estimation of the relative risk of disease progression, evaluated by combining the parameters evaluated in the study, shows that the highest relative risk is for the

combination of IL-1beta and the presence of A. actinomycetemcomitans bacterial species.

- The most harmful combination regarding the biomarkers measured in the study is between PPD and salivary 8-OHdG, leading to a progression of 17.41 times higher in patients with aggressive periodontal disease.
- Regarding the correlation between gingival sulcus depth and the amount of salivary 8-OHdG in healthy subjects, it supports the possibility of using this biomarker in early diagnosis of periodontal disease and to conduct a population screening.

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4. "Salivary Interleukin-1 and 8-Hidroxydeoxyguanosine and their relation with the periodontal status", Aureliana Caraiane, Victoria Badea, Florin Ciprian Badea, **MIRCEA GRIGORIAN**, Romanian Journal of Oral Rehabilitation, Vol. 6, No. 2, April-June 2014, pp. 8-13;
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MEMBER IN RESEARCH PROJECT IN THE THESIS THEME

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