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PhD THESIS

IMPLICATIONS OF VITAMIN D IN THE ETIOPATHOGENESIS OF PERIODONTAL DISEASE

ABSTRACT

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CONTENTS

List of published papers VI
Lis of abbreviations VII
Index of Figures VIII
Index of Tables XI
INTRODUCTION XIV

GENERAL PART 1
1. Periodontal disease 1
1.1. Generalities 1
1.2. Etiology of periodontal disease 2
1.2.1. Infectious agents 4
1.2.1.1. Characteristic microbiological aspects of periodontal disease 4
1.2.1.2. Biofilm of bacterial plaque 9
1.3. Sistemic factors involved in the etiopthogenesis of periodontal disease 12
1.3.1. Hormonal factors 12
1.3.2. Nutritional deficiencies 12
1.3.3. Metabolic diseases 13
1.3.4. Drug factors 14
1.3.5. Emotional factors 14
1.3.6. Genetic factors 15
2. Vitamin D 17
2.1. Generalities 17
2.2. 1-Alpha, 25-dihydroxycalciferol - manner of action 19
2.3. Daily necessary 22
2.4. Deficit of vitamin D_3 - correlated with a multitude of conditions 22
2.4.1. Deficit of vitamin D in periodontal disease and dental caries 23
2.4.2. Deficit of vitamin D in autoimmune diseases 25
2.4.3. Deficit of vitamin D in infections 26
2.4.4. Deficit of vitamin D in neurological diseases 27
2.4.5. Deficit of vitamin D in metabolic diseases 28
2.4.6. Deficit of vitamin D in malignant diseases 28
2.5. Receptor of vitamin D (VDR 29
2.6. Polymorphisms of vitamin D receptor 31
2.6.1. FokI Polymorphism (rs2228570) 32
2.6.2. BsmI Polymorphism (rs1544410) 33

2.6.3. ApaI Polymorphism (rs7975232)....34
2.6.4. TaqI Polymorphism (rs731236)....34

SPECIAL PART

1. Aim and objectives of study....36
1.1. Aim of study....36
1.2. Objectives of study....36
2. Considerations regarding the association of vitamin D receptor polymorphism and chronic marginal polymorphism....39
2.1. Introduction....39
2.2. Aim of the study....41
2.3. Material and method....42
2.3.1. Periodontal clinical examination....44
2.3.2. Extraction and determination of vitamin D receptor genotype....45
2.3.3. Statistical analysis....51
2.4. Results....52
2.5. Discussions....71
3. Considerations regarding the association of low serum level of vitamin D_3 with chronic marginal periodontitis....73
3.1. Introduction....73
3.2. Aim of the study....73
3.3. Material and method....73
3.3.1. Clinical periodontal parameters....76
3.3.2. Quantitative determination of serum level of vitamin D_3....79
3.3.3. Statistical analysis....80
3.4. Results....81
3.5. Discussions....86
3.6. Conclusions....87
4. Considerations regarding the prevalence of periodontal pathogens: Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythensis, Treponema denticola and Prevotella intermedia in patients with chronic marginal periodontitis....88
4.1. Introduction....88
4.2. Material and method....89
4.3. Statistical analysis....96
4.4. Results....97
4.5. Discussions....105
4.6. Conclusions....107

FINAL CONCLUSIONS....108
PERSONAL CONTRIBUTIONS....110
BIBLIOGRAPHY....113
ANNEXES....I

KEYWORDS: chronic marginal periodontitis, vitamin D_3, polymorphism, vitamin D receptor

ABSTRACT OF THE DOCTORAL THESIS

THE GENERAL PART treats systematically data referring to periodontal disease and the implications of vitamin D in the etiopathogenesis of this condition.

Periodontal disease is described in chapter 1 as an infectious disease that results as a consequence of inflammation of the support tissue of teeth, progressive loss of epithelial attachment and alveolar bone.

Periodontal disease is a multifunctional entity in which the bacteria, host and environmental factors interact between them. The factors that contribute to periodontal disease include: bacterial plaque, dental calculus, type of microbes, immune response, systemic diseases, genetic variants, etc.

This condition can appear only when an imbalance is produced between the microbial flora at the periodontal level and the body's own defense mechanism, imbalance produced through the numeric increase in bacteria and their virulence, as well as through the onset of favoring systemic conditions, which however would not be periodontally pathological in the absence of a favoring bacteriologic spectrum.

Bacteria are organized in the shape of a periodontal biofilm, colonizing root surfaces and periodontal pockets surfaces, avoiding the host's defensive mechanisms and directly altering the periodontal tissue. The biofilm consists of microbial cells incorporated in the extracellular matrix that contains substances such as proteins, polysaccharides and nucleic acids. The organizing of microorganisms in biofilm grants them properties that cannot be found in the case of individual bacteria colonies, grown independently.

Vitamin D is presented in chapter 2. The manner of action of the active metabolite of this vitamin – 1 Alpha, 25 dihydroxycalciferol – is described in detail, as well as the correlation between vitamin D_3 deficit and a multitude of conditions: autoimmune diseases, infections, neurological, metabolic, malignant diseases, as well as oral conditions such as periodontal disease and dental caries. Also, the vitamin D receptor (VDR) is highlighted, as well as the polymorphisms of the vitamin D receptor, which can be a risk factor or a protective factor for a certain condition, depending on the gene influenced.

THE SPECIAL PART presents, in a systematic and concise manner, the objectives and design of the study, methods and instruments of investigation, results obtained, discussions and conclusions of three studies, having as a common element the periodontal disease and various etiopathogenetic aspects of vitamin D_3.

The objectives of the study were:

- To identify an association between the polymorphisms of the vitamin D receptor and chronic marginal periodontitis
- To identify certain associations between serum levels of vitamin D_3 and patients with chronic marginal periodontitis, compared with patients without periodontal involvement.
- To determine the prevalence of five periodontal pathogens: *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythensis, Treponema denticola* and *Prevotella intermedia,* in patients with marginal periodontitis, as well as their association with low serum levels of vitamin D.

The objectives of the study were materialized in three clinical, prospective, multicentric studies, whose results were published in specialty journals.

1. CONSIDERATIONS REGARDING THE VITAMIN D RECEPTOR POLYMORPHISM AND CHRONIC MARGINAL PERIODONTITIS

The vitamin D receptor (VDR) gene polymorphism regulates the mineral density and bone transformation. There is a hypothesis that if the polymorphism of the vitamin D receptor (VDR) gene polymorphism influences the gene expression, this polymorphism could play a role in the pathogenesis of periodontal disease with bone tissue involvement.

AIM OF THE STUDY: To add to the low number of studies on this topic in the Caucasian population, this study aims to investigate whether the VDR gene polymorphism is associated with CP in a population in Western Romania, by determining the prevalence of the *Bsm*I (rs1544410), *Apa*I (rs7975232), *Taq*I (rs731236) and *Fok*I (rs2228570) genotypes and comparing the CP group with the periodontally healthy group.

MATERIALS AND METHODS: This case-control study involved a Western-Romania population including 100 patients (53 patients with CP and 47 healthy patients), all Caucasians. The patients were selected from the outpatients of a large dental clinic in Western Romania and were treated in the Department of Periodontology of the "Victor Babes" University of Medicine and Pharmacy of Timisoara during 2013-2016. All patients understood the purpose of the study and agreed to receive a clinical examination and provide biological samples. The study was approved by the Ethics Commission of the University of Medicine and Pharmacy Timisoara (No10/2013), Romania, and was performed in accordance with guidelines of the Declaration of Helsinki (2013). Included in the CP group were patients from with CAL of at least 4 mm at more than one tooth or more than two interproximal sites with PD > 5 mm (27). Included in the control group were patients with no CAL or bone loss, which was confirmed radiologically, PD < 3 mm, absence of any clinical signs of periodontitis and no previous history of periodontal disease.

The exclusion criteria were: patients with diabetes, pregnant/lactating patients, patients who frequently used anti-inflammatory drugs during the last 3 months, patients who used immunosuppressive drugs, and patients with cardiovascular failure, renal failure, respiratory failure, or autoimmune diseases. Only non-smokers with a negative smoking history were included in this study.

Periodontal clinical examination. All patients were examined by the same periodontologist (DR). The measured periodontal parameters included: the plaque index (PI, Silness & Loë 1964); bleeding on probing (BOP); probing depth (PD), which was recorded as the mean overall value (mean PD) and the mean value of the deepest PD of the mouth (max PD); the gingival clinical attachment level (CAL) calculated as a difference between Probing Depth (mm) and gingival margin Recession (REC)(mm); and the number of lost teeth. Digital panoramic radiographs were captured. At the end of the periodontal examination, 2 ml of venous blood were collected from each examined patient in a K2-EDTA tube and were sent to the laboratories of the Biochemistry Department of the "Victor Babes"

University of Medicine and Pharmacy Timisoara, to determine the serum vitamin D levels and polymorphisms in the VDR gene.

Extraction and determination of the vitamin D receptor genotype. Genotypes for the 4 VDR SNPs (rs1544410, rs2228570, rs731236, and rs7975232) were assessed using a TaqMan probe and primer sets (ThermoFisher Scientific, Waltham, MA, USA) specifically designed for the allele of interest.

All **statistical analyses** were conducted using the R software, version 3.2.4, R Core Team (2016). Inter-group comparisons were performed using the Mann-Whitney test for quantitative variables. Contingency tables for categorical variables were analysed using chi-square tests. Odds ratios were estimated using logistic regression models. The models studied the association between the presence of CP and VDR polymorphisms. The variables that showed significant differences between the patient and control groups in the univariate analyses (i.e. patient age and serum level of vitamin D) were used as covariates in the extended models.

RESULTS. There was a relevant association between *Fok*I (rs2228570) polymorphism and CP. The subjects with the CC genotype for this SNP were 19 times more likely to have the disease (OR 19.58, 95% CI 2.67 – 198.92) and with TC genotype almost 8 times (OR 7.86, 95% CI 1.29 – 61.56) than subjects with TT genotype as per adjusted regression analyses (table 3). Additionally, the presence of the C genotype increased the susceptibility to CP (p=0.030) as shown in table 2.

In cases with SNP rs1544410 (*Bsm*I), heterozygous carriers were nearly four times more likely to have CP (OR 3.76, 95% CI 1.15-13.80), as per crude regression analyses (Table 4). This shows that AG genotype is a risk factor for CP, independently of age and serum level of vitamin D. No association was detected between homozygous carriers of the variant allele SNP rs1544410 and CP.

No significant association was detected between the *Taq*I (rs731236) SNP and CP (Table 2, 5)**,** nor between the *Apa*I (rs7975232) polymorphism and CP (Table 2, 6).

CONCLUSIONS. The presence of the CC and CT genotype of the *Fok*I polymorphism (rs2228570) and the AG genotype of the *Bsm*I polymorphism (rs1544410) seems to predispose individuals to CP; no associations between these two SNPs were identified in the Western-Romania population.In our study, vitamin D levels were lower in CP-patients compared with the controls.

Determining whether the VDR polymorphisms variations and the serum vitamin D levels are associated with CP supports a re-evaluation of the pathogenic factors of CP, thereby improving the quality of prophylaxis and therapies.

2. CONSIDERATIONS REGARDING THE ASSOCIATION OF LOW SERUM LEVEL OF VITAMIN D_3 WITH CHRONIC MARGINAL PERIODONTITIS

During the last decade, an increased attention was granted to the role of 1.25-dihydroxy vitamin D3 ($1.25(OH)_2D_3$) – the active form of Vitamin D – in inflammatory, infectious and autoimmune diseases. Low serum vitamin D levels have been associated with

a multitude of diseases, such as osteoarticular, metabolic, cardiovascular, digestive, endocrinal, neurologic and psychiatric.

There are a few studies which have found an association between the decreased level serum of 25(OH)D and periodontal infection.

AIM OF THE STUDY was to explore the association between low serum levels of $1.25(OH)_2D_3$ and chronic periodontitis (CP).

MATERIALS AND METHODS. This case-control study included 56 patients with CP (29 women, 27 men) and 56 healthy periodontally patients (33 women, 23 men). All participants were patients selected among the outpatients of the Department of Periodontology of the "Victor Babeş" University of Medicine and Pharmacy in Timisoara, between 2014 and 2016.

The criteria for exclusion were: pregnancy, diabetes, hepatitis, cancers, HIV, intake of anti-inflammatory and immunosuppressive drugs.

All patients were examined by a single clinician. The clinical parameters of periodontal disease measured in the present study included the plaque index (PI, Silness & Loë 1964), periodontal probing depth (PD), bleeding on probing (BOP); periodontal attachment level (CAL) and the number of absent teeth (AT). Clinical measurements were recorded on the online periodontal chart of the Department of Periodontology of the University of Bern. The quantitative determination of serum levels was performed with an ELISA (enzyme-linked immunosorbent assay) kit for Vitamin D3 (MyBiosourse, San Diego, CA, USA).

STATISTICAL ANALYSIS were conducted using the R software, version 3.3.2, R Core Team (2016). Inter-group comparisons for interval and ordinal variables were performed using the Mann–Whitney tests (unless otherwise specified), due to lack of normality of the data. Chi-square tests were used to compare proportions. Associations between vitamin D level and clinical parameters of the study subjects were assessed by computing Spearman's rank correlation coefficients. Finally, in order to investigate the influence of vitamin D level on periodontal health status, a binary logistic regression model was considered, with the presence of periodontal disease as outcome variable, and vitamin D level and patient age as predictors.

RESULTS. The vitamin D serum level of patients (13.01±5.10 ng/ml) was significantly lower than in the control group (22.10±5.63 ng/ml) (p <0.001). All clinical parameters, with the exception of PII, were highly positively correlated with each other and negatively correlated with the vitamin D level. It was estimated that the odds of developing periodontitis increase by 6.7% for each one year increase in age, and drop by 29.6% for each one unit increase in vitamin D level.

CONCLUSIONS. The mean serum level of $1.25(OH)_2D_3$ in patients with CP was almost half of the mean level in controls.

The following periodontal parameters are negatively correlated with vitamin D serum level: PD, CAL, BOP. The probability of developing CP increases by nearly 7 % with every year of age. The chances of developing periodontitis decrease with almost 30 % for every increase of 1ng/ml of serum level of vitamin D.

3. CONSIDERATIONS REGARDING THE PREVALENCE OF PERIODONTAL PATHOGENS: *AGGREGATIBACTER ACTINOMYCETEMCOMITANS, PORPHYROMONAS GINGIVALIS, TANERELLA FORSYTHENSIS, TREPONEMA DENTICOLA* AND *PREVOTELLA INTERMEDIA* IN PATIENTS WITH CHRONIC MARGINAL PERIODONTITIS

Chronic periodontitis debut is influenced by the simultaneous activity of multiple factors that initiate the disease and its progression: the virulence of periodontal pathogens, the local conditions and the susceptibility of the host. The periodontal pathogens best documented so far are *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythensis, Prevotella intermedia, Fusobacterium nucleatum, Campylobacter rectus, Eikenella corrodens, Peptosreptococcus micros, Eubacterium(2)*. The presence of periodontal pathogens seems to cumulate with the immune response of the host, the result being the periodontal destruction. Classical experiments have shown that accumulation of bacterial plaque on dental surfaces results in an inflammatory gingival response in the adjacent gingiva, whereas removing the bacterial plaque leads to regression of symptoms of gingival inflammation (1). It is well-known nowadays that more than 500 various species can colonize the oral cavity, so that each individual may host up to 50-150 various species. (1)

THE STUDY AIM was to analyze the quantitative determination of five periodontal pathogens, namely of the "purple" complex *Aggregatibacter actinomycetemcomitans,* of the "red" one *Porphyromonas gingivalis, Tanerella forsythensis, Treponema denticola,* and of the "yellow" one *Prevotella intermedia,* as well as their associations in patients with chronic periodontitis.

MATERIALS AND METHODS. This case-control study included 110 patients from the Western Romania, 60 out of them with chronic periodontitis (29 women) and 50 healthy periodontally healthy (32 women). Patients were selected and treated in the Department of Periodontology of the "Victor Babeş" University of Medicine and Pharmacy in Timisoara, between 2013 and 2016.

The criteria of exclusion were: diabetes, hepatitis, HIV, malignant tumors, prolonged treatment with anti-inflammatory drugs, immunosuppressive drugs, pregnancy and lactation.

Patients were examined by two specialists (DR, DM). A Williams probe with gradations at 1, 2, 3, 5, 7, 9 and 10 mm was used. The periodontal parameters recorded were: the plaque index (PI, Silness & Loë 1964); the full-mouth bleeding on probing score (BOP); the probing depth (PD), which was recorded as the mean overall value (mean PD) and the maximum PD value of the mouth (maxPD); the gingival clinical attachment level (CAL); the number of absent teeth (AT). Clinical measurements were performed at six sites per tooth. All data were recorded on the online periodontal chart of the Department of Periodontology of the University of Bern.

The microbiological samples from the gingival sulcus to detect the periopathogens *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythensis, Treponema denticola, Prevotella intermedia* were taken with sterile paper cones by inserting them for 10 seconds in the gingival sulcus. A sample was taken from each patient from the

control group, from 6 sites with PD > 4 mm. The samples were collected from the controls in 6 random sites. The paper cones were introduced in sterile recipients and transported to the laboratory of the Biochemistry Department of the University of Medicine and Pharmacy in Timisoara, in order to identify the periodontal pathogen markers through the polymerization chain reaction (PCR).

Extraction of nucleic acids was performed from the collected crevicular fluid, using the Qiagen kit (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany**)**. The samples were amplified for micro-Ident. Hybridization and the reaction with alkaline phosphatase were performed on a paper band. Data were introduced in the table provided by the micro-IDent kit, (Hain Lifesciences, Nehren, Germany) and were interpreted according to the indications of the producer.

STATISTICAL ANALYSIS. The patient was considered the unit of analysis in this study. Inter-group comparisons in the were performed using the Mann – Whitney test (for two groups) and Kruskal – Wallis test (for more than two groups), respectively. Contingency tables for categorical variables were analysed using the chi square test or the Fisher's exact test, where appropriate. A *p*-value < 0.05 was regarded as statistically significant. When multi-group comparisons showed the existence of significant differences between the analysed groups, they were followed by pairwise post-hoc tests using the Bonferroni corrections. Odds ratios describing associations between microbial species were computed using logistic regression models.

RESULTS

For the periopathogens *Pg, Pi, Tf* and *Td*, the proportion of individuals for whom the investigated microbial species were present was significantly higher for periodontitis patients than for controls, but does not differ between MCP and SCP groups. In comparison, for *Aa*, the detection frequency is slightly lower in the control group than in the patient groups, however this difference is not statistically significant.

The detection scores of the five microbial species were all pairwise positively correlated, and all these correlations were statistically significant, the strongest correlation was found between *Pg, Tf* and *Td*.

The detection scores for the analyzed species are negatively correlated with the serum level of vitamin D. The correlation is significant in the case of Pg, Tf, Td and Pi and only marginally significant for Aa.

CONCLUSIONS

- All species of periopathogens investigated were detected more frequently in patients with CP than in the control group.

- The quantitative values of periopathogens were higher in patients with CP, when compared with the patients without this condition.

- There were no significant differences of the quantitative values of periopathogens in the group of patients with moderate chronic periodontitis, when compared patients with severe chronic periodontitis.

- A significant correlation of periopathogens was found between *Tf, Td* and *Pg,* belonging to the "red" complex. Thus, the presence of *Tf* in the gingival sulcus raises the chances of detection of *Pg* more than 40 times, in comparison with healthy individuals. Also, it seemed that the presence of *Td* increases the risk for infection with *Tf* over 35 times.

- Quantitative determination of periopathogens using the PCR technique is useful for the prescription of antibiotic regimens in chronic periodontitis, resulting in an efficient treatment.

MAIN CONCLUSIONS outlined in this thesis are:

1. The purpose of the study was to determine the implications of vitamin D in the etiopathogenesis of periodontal disease.

2. The objectives of the study were to identify an association between the genetic polymorphism of the vitamin D receptor, serum level of vitamin D, the level of the main periodontal pathogens and chronic marginal periodontitis in the Western Romania population.

3. In the case of investigating the polymorphisms of vitamin D, four polymorphisms were analyzed: BsmI (rs1544410), ApaI (rs7975232), TaqI (rs731236) and FokI (rs2228570). We identified a significant association between the FokI polymprhism (rs2228570) and chronic periodontitis. In the case of this polymorphism, the subjects with CC genotype risk an approximately 19 times greater incidence, while those with TC genotype an approximately 8 times greater one. In the case of the Bsml genotype (rs1544410), the heterozygotic carriers have a four times greater risk of developing chronic periodontitis.

4. The analysis of the serum level of vitamin D showed that the serum level of vitamin D in patients with chronic periodontitis (13.05±5.1 ng/ml) was significantly lower than in the control group (22.10±5.63 ng/ml) ($p<0.001$). Moreover, a low level of vitamin D was also associated with an increase in the values of inflammatory markers of periodontal disease, especially with probing depth and attachment loss, as these are indicators of the degree of severity of periodontal disease.

5. Investigating the periodontal pathogens included the species: *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythensis, Treponema denticola* and *Prevotella intermedia.* All species investigated were detected more frequently and in larger quantities in patients with marginal chronic periodontitis than in patients in the control group. A significant quantitative correlation was identified between *Tanerella forsythensis, Treponema denticola* and

Porphyromonas gingivalis. The presence of one of these three increases tenfold the chances of becoming infected with the other two as well.

6. The present study represented a complex approach of the factors involved in the etiopathogenesis of periodontal disease. An association was proved between periodontal disease and the low serum level of vitamin D, between periodontal disease and certain genotypes of the vitamin D receptor. The profound involvement of vitamin D in the good functioning of the immune system explains why the quantitative modifications of the serum level of vitamin D, as well as the qualitative modifications of the VDR genotype, create a vulnerability of the immune defense at the periodontal level, a fact explained quantitatively and qualitatively by the periodontal pathogenic species.

7. The study has also practical applications of investigation and therapy. Thus, the investigation of the serum level of vitamin D must be followed by D_3 supplementation, for the purpose of preventing the apparition of periodontal disease, as well as for its treatment. Investigating the VDR a genotype at the FokI (rs2228570) and BsmI (rs1544410) levels has a special value in preventing of the apparition of periodontal disease by adopting adequate prophylactic measures. The detection of the periodontal pathogenic species though genotyping allows to rapidly establish a personalized antibiotic treatment.

8. The complex study of the etiopathogenesis of periodontal disease can also be utilized in the case of the etiopathogenesis of other conditions that affect the dental apparatus, with the possibility to identify certain common factors triggering various conditions at the dental level.