GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

- HABILITATION THESIS -

PERIODONTAL DISEASES FROM DIAGNOSIS TO COMPLEX TREATMENT

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CONTENTS

Abbreviations

GCF – Gingival crevicular fluid
PMN – polymorphonuclear neutrophils
MMP – matrix metalloproteinases
TNF – tumor necrosis factor
Il – interleukin
MIP-1a – macrophage inflammatory protein-1a
PCR – protein chain reaction
GAP – generalised aggressive periodontitis
SCP – severe chronic periodontitis
RANKL – receptor activator of nuclear factor kappa-B ligand
CRP – C-reactive protein
CVD – cardiovascular diseases
CKD – chronic kidney diseases
GFR – glomerular filtration rate
ESRD – end stage renal diseases
RA – reumatoid arthritis
ICID – International Center of Immunodeficiency diseases
AAA – abdominal aortic aneurysm
PD – periodontal diseases
Pg – Porphyromonas gingivalis
Aa – Aggregatibacter actinomycetemcomitans
Fn – fusobacterium nucleatum
BMI – body mass index
LPS – lipopolysaccharide
CHX – chlorhexidine

THESIS SUMMARY

The habilitation thesis entitled ”Periodontal diseases from diagnosis to complex treatment” reflects study and research activities from a period of over 15 years in my career and represents the synthesis of two of my postdoctoral scientific research directions oriented towards current ways of diagnosing periodontal disease in the context of complex biochemical and immunological interrelationships with various local or systemic pathologies or circumstances and their treatment in this complex context.

According to the recommendations of the National Council for Attestation of University Degrees and Certificates (CNATDCU) and according to the order of the Ministry of Education and Scientific Research no. 3121/2015, we structured the habilitation thesis in three sections, presented below.

Section I, after exposing a selection of my achievements in the medical professional activity, in the academic one and in the scientific research, presents the main study directions to which I contributed and the synthesis of the most important 29 articles published in specialized journals indexed both in Thomson ISI Web of Science Core Collection (26), as well as in international databases (3).

The scientific research activity following the defense of the doctoral thesis (April 2006), continued the research carried out within the doctorate and expanded in the directions dedicated to exploring current methods of diagnosis of periodontal disease in the context of complex interrelationships with various systemic diseases, and evaluating the effectiveness of instrumental methods and of the adjunctive treatment methods of periodontal treatment. Each chapter lists the most relevant publications in the field.

Chapter I – ”*Modern diagnostic methods in periodontal disease*” is a comprehensive research direction that brings together the results of personal research on saliva as a potential source of extraction of amplifiable genomic DNA by PCR method, the value of gingival crevicular fluid in the evaluation of bacterial associations involved in the periodontal disease and finally explores a number of particular situations that require both complex and accurate diagnosis, such as the periodontal evaluation in cases of orthodontic treatment, the complex diagnosis of endo-periodontal lesions, and the complex diagnosis in cases of association of periodontal disease with a number of systemic pathologies such as cardiovascular disease, chronic kidney disease, osteo-articular siseases and hepatitis C.

Chapter II – ”*Complex treatment of patients with periodontal sisease*” brings together four main research directions that investigate: the effects of root planning with reciprocating intruments, the effects of conventional non-surgical periodontal therapy in patients with systemic diseases, the contribution of adjunctive therapeutic methods in the complex treatment of periodontal disease and the influence of systemic therapies on periodontal status in patients with systemic disorders.

Section II details the future development plans for teaching, research and academic careers.

In terms of teaching, I propose the development of the simulation component in the activity of students and residents in the newly established Simulation Center, aimed at training and developing periodontology instrumentation skills at the student level and practicing various periodontal surgery techniques, skills that is addressed to residents in the periodontology specialty that I coordinate.

Academically, in addition to completing the teaching activity and projects for making taxtbooks for students and residents, I propose to organize training courses in the field of periodontology.

In the scientific research I have in mind the co-optation of PhD students interested in the study of complex diagnosis and integrated therapeutic management of periodontal disease presented in this thesis, but also in other research directions in which I have been involved over the years.

Section III represents the scientific support of my research activity so far and the basis for my future research plans and projects. This section contains a list of relevant bibliographical references related to the studies presented in the habilitation thesis.

REZUMATUL TEZEI

Teza de abilitare intitulată *”Bolile parodontale de la diagnostic la tratament complex”* reflectă activități de studiu și cercetare dintr-o perioadă de peste 15 ani din cariera mea și reprezintă sinteza a două dintre direcțiile mele de cercetare științifică postdoctorală orientate către modalități actuale de diagnostic al afecțiunilor parodontale în contextul interrelațiilor complexe biochimice și imunologice cu diferite patologii sau circumstanțe locale sau sistemice și tratamentul lor în acest context complex.

Conform recomandărilor Consiliului Național pentru Atestarea Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU) și ale ordinului Ministerului Educației și Cercetării Științifice nr. 3121/2015, am structurat teza de abilitare în trei secțiuni, prezentate în continuare.

Secțiunea I, după expunerea unei selecții a realizărilor mele în activitatea profesională medicală, în cea academică și în cercetarea științifică, prezintă principalele direcții de studiu la care am contribuit și sinteza celor mai importante 29 articole publicate în jurnale de specialitate indexate atât in Thomson ISI Web of Science Core Collection (26), cât și în baze de date internaționale (3).

Activitatea de cercetare științifică consecutivă susținerii tezei de doctorat (aprilie 2006), a continuat cercetările derulate în cadrul doctoratului și s-a extins în direcții dedicate explorării unor metode actuale de diagnostic al bolii parodontale în contextul interrelațiilor complexe cu diferite afecțiuni sistemice, și evaluării eficienței metodelor instrumentale de tratament parodontal și a metodelor adjuncte de tratament. În cadrul fiecărui capitol sunt menționate cele mai relevante publicații în domeniul abordat.

Capitolul I – ”Metode moderne de diagnostic în boala parodontală”, se constituie într-o direcție comprehensivă de cercetare care reunește rezultatele unor cercetări personale referitoare la salivă ca potențială sursă de extracție a AND-ului amplificabil genomic prin metoda PCR, la valoarea fluidul gingival crevicular în evaluarea asocierilor bacteriene implicate în boala parodontală și explorează, în final, o serie de situații particulare care necesită un diagnostic atât complex cât și precis, precum evaluarea parodontală în cazurile de tratament ortodontic, modalități complexe de diagnostic al leziunilor endo-parodontale, și diagnosticul complex în cazurile de asociere a bolii parodontale cu o serie de patologii sistemice precum afecțiunile cardiovasculare, boala renală cronică, afecțiunile osteo-articulare și hepatita C.

Capitolul II – ”Tratamentul complex al pacienților cu afecțiuni parodontale” reunește patru direcții principale de cercetare care investighează: efectele netezirii radiculare cu instrumente cu mișcare reciprocă, efectele terapiei parodontale convenționale ne-chirurgicale la pacienții cu afecțiuni sistemice, contribuția metodelor terapeutice adjuncte în tratamentul parodontal complex și influența terapiilor sistemice asupra statusului parodontal la pacienții cu afecțiuni sistemice.

În **Secțiunea II** sunt detaliate planurile de dezvoltare viitoare cu privire la activitatea didactică, la activitatea de cercetare și la cariera academică.

În planul ativității didactice îmi propun dezvoltarea componentei de simulare din activitatea studenților cât și a rezidenților în cadrul Centrului de Simulare nou înființat, vizând formarea și dezvoltarea de abilități de instrumentare în parodontologie la nivel de student cât și practicarea variatelor tehnici de chirurgie parodontală, competențe care se adresează rezidenților din specialitatea de parodontologie pe care o coordonez.

În plan academic, pe lângă perfectarea activității de predare și proiectele de realizare de manuale pentru studenti și rezidenți, îmi propun organizarea unor cursuri de perfecționare în domeniul parodontologiei.

În cadrul cercetării științifice am în vedere cooptarea doctoranzilor interesați de studiul diagnosticului complex și al managementului terapeutic integrat al bolii parodontale prezentate în această teză de abilitare, dar și pe alte direcții de cercetare în care m-am implicat de-a lungul anilor.

Secțiunea III reprezintă suportul științific al activității mele de cercetare până în prezent și baza pentru planurile și proiectele mele viitoare de cercetare. Cuprinde o listă de referințe bibliografice aferente studiilor și lurărilor prezentate în cuprinsul tezei de abilitare.

SECTION I

OVERVIEW OF PROFESSIONAL, ACADEMIC AND SCIENTIFIC ACTIVITY

Career, as a supposedly natural encounter between vocation and opportunities, is actually a complex process with different tasks. My career path has been best clarified when I was able to fully embrace the developmental task of relating knowledge of myself to the knowledge of my main 3 occupations: practicing stomatology and teaching it, intertwining with research.

According to humanists, individuals are career mature or ready to make appropriate choices when they have engaged in planful exploration and have appropriate occupational knowledge, self-knowledge, and decision-making knowledge. Looking upon the milestones of my evolution, I am now ready to acknowledge the challenges ones has to undergo in order to achieve such a vocational maturity, as well integrating the fact that this should be a continuous process.

Having had the context to meddle the 3 main areas, teaching, practicing and researching, I also had the opportunity to develop myself professionally in a multicultural, multifunctional environment that stimulated the expansion of critical abilities, in the spirit of a stable set of values that I rely on: *high moral and professional conduct, mutual respect, sensitivity for human diversity and cultural anchors.* The overview of the main milestones achieved includes as follows:

A. Academic activity

My didactic activity encompasses 30 years of sustained effort within the framework of the same main discipline, initially called Odontology and Periodontology, while later on, with the emerge of new specialties, got divided and I continued with Periodontology.

The milestones of this evolution are as follows; two weeks only after graduating the Faculty of Stomatology in 1991, I started the teaching career as a *junior assistant teacher* within the Department of Odontology and Periodontology, The Faculty of Stomatology, Iași. Early on, I could contribute to the realization of the very first manual of Periodontology, published by the Litografia UMF Iasi, under the coordination of late professor Radu Vataman, thus opening a very consistent line of publications that later unfolded. Four years later, my results allowed the participation in the contest for the *assistant professor position*; within the next 7 years (1995-2002), I was involved in the discipline research team, actively contributing to the common projects, thus leading to my first presentations of research results in the congresses and conferences related to Cariology, Endodontics, Periodontology, on overall of 38 oral presentations and 21 published articles in indexed journal and conference volumes. In between 1999-2000 I was co-author to:

1.Endodonţie practică, C-ţa Mocanu, Maria Vataman, colab. I. Ichim, S. Andrian, Sorina Solomon, Editura “Apollonia”, Iaşi 1999.

2.Caria dentară – Protocoale şi tehnici, S. Andrian, Şt. Lăcătuşu, colab. Gianina Iovan, Angela Ghiorghe, Sorina Solomon, C. Topoliceanu, I. Ichim, V. Dănilă, Editura "Apollonia" – Iaşi, 1999.

3.Parodontologie clinica, Silvia Mârtu, Constanta Mocanu, Editura "Apollonia" – Iaşi, 2000

The next significant period, as a lecturer, (2001-2016), is marked by important achievements that contributed to the structuring of the Periodontology field in our Faculty: the full development of new courses, such as the Periodontology module within the modular program of *Complex oral rehabilitation* (sixth year dental medicine), *Periodontal pathology in HIV positive patient* (fourth year dental medicine), *Periodontology and periodontics for the patient with special needs* (sixth year dental medicine) periodontal course for the series of stomatology nurses, *Periodontology and splinting devices* for the Dental Technique Collegium. The advanced English speaking and writing skills have also made possible the initiation and implementation of a Periodontology discipline fully taught in English, series that are continuing to the present study cycles. Relating to my constant preoccupation to structure and deliver up to date information to our students, the type of knowledge that best meets their needs and follows the accredited programs, I configured manuals and materials to be used in the teaching process that were uploaded to the E-learning platforms/ Microsoft Teams directories for open access, and as well several important hardcopy volumes:

1. Clinical periodontology. Ed."Gr.T.Popa", U.M.F. Iasi, 2015

2. Periodontology Propaedeutics. Ed."Gr.T.Popa", U.M.F. Iasi, 2013

3. Management clinic, imagistic si biologic in boala parodontala. Ed."Gr.T.Popa", U.M.F. Iasi, 2011

4. Ghid practic de propedutica parodontala. Ed. PIM Iasi, 2010

A significant part of my academic activity during this period is connected to the facilitation of practical skills development for our students, a critical aspect of their long-term medical professionalization. As such, I coordinated, deisgned and revised the activities for:

a. Practical activities in the discipline of Cariology, with the students of the Faculty of Dentistry, year VI: Complex oral rehabilitation (2000-2004);

b. Practical activities in the discipline of Periodontology, with the students of the Faculty of Dentistry, year VI: Management of patients with disabilities (2002-2005);

c. Practical activities in the discipline of Periodontology, with the students of the Faculty of Dentistry, year VI: Juventostomatology (2002-2005);

d. Practical activities in the discipline of Periodontology, with the students of the Faculty of Dentistry, year IV: Management of AIDS patients (2002-2005);

e. Practical work in the discipline of Periodontology with the students of the College of Nursing Assistants, year III (2001-2004) (according to the norm, on an hourly or voluntary basis to cover the vacant norms);

f. Practical work in the discipline of Periodontology with the students of the College of Dental Technique, year III (2002-2010) (according to the norm, on an hourly or voluntary basis to cover the vacant norms);

g. Practical work in the discipline of Periodontology, with the students of the Faculty of Medical Bioengineering, year VI (2002-2005) (according to the norm, on an hourly or voluntary basis to cover the vacant norms).

Ever since 2016, as an associate professor, I had the honour and consistent responsibility to extend the didactic activity by including the managerial and professional tasks related to the position of Residency Coordinator (October 2019 – present), Interim Department Chair (October 2019- March 2020) and Head of Periodontology Discipline (October 2019 – present); the associate challenges alongside the global quest for responses due to the COVID-19 pandemics have elicited the refinement of organizational abilities, time management and resource planning.

All along these 30 years, I actively encouraged students in participating in clinical, academic and research activities leading to various papers that I coordinated and were then presented in different scientific events, which in turn lead to them receiving prizes. As well, have coordinated over **60** license theses for dental medicine students from the Romanian, English, and French sections. Also, I promoted and guided the active participation of students in scientific student manifestations and workshops. Within the periodontology discipline, I taped on VHS support live demonstration of different instrumental procedures in periodontology such as splinting techniques and a consistent portfolio of clinical study cases presentations. As well, I organized and coordinated teams of students, trainees and residents who contributed to the data collection and recording in the *WHO-2002 Iaşi* chart within the Oral health in Romania screening with the subsection "Oral health in pregnant women" and "Oral health of the HIV positive patient". These teams went to the HIV-positive ward of the University Hospital for Infectious Diseases - Iaşi and to the "Cuza Vodă" and "Elena Doamna" Maternity, respectively, thus managing to ensure both a good interclinical collaboration and homogeneous work teams if efficient.

Regarding the interest for the development of the didactic component of my activity, during November-December 2003 I had the opportunity to visit School of Dentistry, University of Louissville, Kentucky, a documentation internship that focused on learning modern ways of teaching in medical education. I also had the opportunity to participate in 3 workshops with hands-on component on following topics: Algo-dysfunctional TMJ syndrome by Professor Jeffrey Okeson, Composite aesthetic restorations of the cervical third (the topic later developed in my doctoral thesis) by Professor Paul Belvedere and Aspects regarding fixed prosthodontics in the cervical third by Professor Herbert T. Shillingburg. I had the great honor to participate directly for 2 weeks in the periodontology residency program coordinated by Professor Henry Greenwell, the 4th listed program in the United States at that time.

The skills I thus acquired, in terms of teaching in medical education with its particularities and challenges, materialized in the establishment of the Laboratory of Teaching Technologies, a department that I coordinated for 3 years and which was equipped with state-of-the-art equipment (special scanner for radiographs, professional camera with special lenses for medical photography, software for image processing and digital presentations, a professional Rank-Xerox multifunctional, state-of-the-art composite materials, instruments, literature, collections of famous dental magazines from last 10 years) donated by School of Dentistry, University of Louisville, Kentucky to the Faculty of Dental Medicine of the University of Medicine and Pharmacy, Iasi. The knowledge and training acquired during the visit to UofL were continuously implemented in my teaching activities,

they were transferred as teaching methodologies to colleagues in the discipline and department and contributed to the modernization of our integrated modules.

Since 2011 (2011-2016 trainer, 2016-2019 teaching, 2019-present coordinator of the residency program), I also became a trainer within the newly established residency program of Periodontology, I had the opportunity to coordinate the practical and theoretical activity of resident doctors, in training, in the specialty of Periodontology, but also of successive generations of resident doctors in training, regardless of the chosen specialization: prosthodontics, endodontics or dento-alveolar surgery. On a permanent basis, I provided my best of knowledge for doctors undergoing training in Periodontology under my coordination, both through lectures, case presentations, and especially through intensive training in practical activity, aiming to pass on the best information and arouse interest in the Periodontology specialty. I especially encouraged the activity of personal documentation for resident doctors, participation in specialization courses within the country and abroad, in specialized congresses, continuous professional training. The resident doctors in the training stage, who have shown interest in scientific and research activity in the field, have been integrated in the research team of the clinic and have the quality of co-author in specialized articles or studies communicated at scientific events.

Throughout my entire university career, I would not have been able to cope with these duties if I had not made diligent, tireless efforts of professional training. I pledged to be constantly up to date with the latest developments in the field of periodontology participating in the Congresses organized by the Romanian Society of Periodontology and UNAS, at the Europerio Congresses of the European Federation of Periodontology.

Overall, my involvement as member of the discipline team and faculty department has led to various acknowledgments of my potential and valuable input:

a. membership in various scientific committees for national and international congresses, conferences (2008- present);
b. membership to national committees that are responsible to create the subjects for the residency exam (2008-2019);
c. membership to local committees responsible for the subjects in the bachelor exam (2017-2020);
d. membership in committees for job openings in medical faculties (Iasi, Craiova) and in the medical system (DSP Iași, DSP Piatra Neamț, DSP Craiova);
e. membership in various grants aimed at further developing the skills of our students beyond the fixed curricula, such as:
    - Expert within the project *Practical training courses for the rapid integration on the labor market of students specialized in dentistry*, POSDRU/90/2.1/S/ 63942. Sectoral Operational Program for Human Resources Development 2007-2013. Priority Axis 2 "Correlating lifelong learning with the labor market", major area of intervention 2.1 "Transition from school to working life";
    - Long-term expert from the applicant in the project POSDRU / 160 / 2.1 / S / 139881, “Professional counselling for students in medicine and integrated practice program in the field of general and dental medicine”;
    - Voluntary participation in the preparation of the necessary documents for the Dent-internship platform (project “Practical training internships for the rapid

integration on the labor market of students specialized in dentistry", POSDRU / 90 / 2.1 / S / 63942);

- voluntary participation in the elaboration of the necessary documents for the Dent-courses platform (project "Adapting the offer of higher dental medical education to the needs of the labor market and of the knowledge-based society" 2007-2013, Project ID: 63699);
- Trainer in oral rehabilitation, (project "Center for training specialists and resources in oral rehabilitation" POSDRU 2007-2013, Project ID: 62208).

B. Professional medical practice

After the enrolment in the university (September 30, 1991), I also started working as a dental assistant in the Clinic of Dentistry and Periodontology in the University of Medicine and Farmacy, Iaşi, within the Polyclinic Dispensary no. 1 and later in the Dental Medical Education Base (BIMS). Based on the free practice authorization no. 1415 (no. Reg. 5124 of 10.XI.1998) and of the decision no. 331 of 30.IV.1998, I then performed as integrated with 0.5 norm the position of general dentist in the Clinic of Dentistry and Periodontology – Dental Polyclinic nr.1, Iași.

According to OMS no. 727 of 07.IX.2000, I provide medical assistance as a primary doctor in general dentistry within the collective agreement with CNAS – Iași. From September 1992 to July 1994 I provided private dental care at the Medical Center "St. Apollonia" no.1, based on the special approval for free practice no. 19638 of 23.IX.1992. From September 1994 to September 1998 I provided private dental care in the "Danident" office, while from October 2002 until now I provide private dental care in the "CMI Sadent" dental clinic.

C. Research and related publishing

As the current thesis will further demonstrate, my research path has been shaped by the global and local rhythm of development in the field of periodontology. When in April 2006, I successfully defended my doctoral thesis, entitled ***"Clinical and therapeutic particularities in patients with loss of substance in the cervical vestibular area"***, under the coordination of Prof. Dr. Ştefan Lăcătuşu, I was honored to get acknowledged for several national premiers: the introduction of SensiTest as device for diagnostics and the use AFM images, the mathematical processing of the collected data that led to a three-dimensional analysis of finite elements, alongside the use of Dental Suite software for radiology processing. What was back then a mere interface in-between the need to further develop diagnostic methods and interventions for the noncarious lesions in close vicinity to periodontal tissues, configured the whole research interests and relevant projects I got involved into in the last 15 years.

As the international scientific community continued to structure the field of periodontology, as reflected above in the academic section of this brief review, I was able to actively contribute to the shaping of the discipline in our Faculty, developing relevant courses and practical activities for students in the final years. The research projects followed naturally to sustain this effort, covering two main directions:

a. modern methods of diagnosis of the periodontal disease in relation to diverse systemic pathologies, encompassing aspects such as the importance of gingival crevicular fluid

in the assessment of bacterial associations involved in periodontal disease, the SEM analysis of the root surfaces biofilm of human teeth with endodontic-periodontal lesions, salivary metalloproteinase-8 and metalloproteinase-9 evaluation in patients undergoing fixed orthodontic treatment, the relationship between periodontal disease and various systemic diseases such cardiovascular diseases, chronic kidney diseases, osteo-articular diseases, chronic viral hepatitis.

b. complex treatment directions including effects of root planning with power-driven reciprocating instruments, effects of non-surgical conventional periodontal therapy in systemically impaired patients, photoactivation therapy, antibiotics in the adjunctive treatment of periodontal disease, desensitizing agents in the treatment of dentin hypersensitivity.

Representative research projects supporting these lines of inquires include:

- During the activity carried out in the Pilot Research and WHO-Collaboration Center, Iaşi I coordinated the activity of two sub-sections: "Oral health in pregnant women" and "Oral health in HIV-infected patients", integral parts of the study on health of the Romanian population ", a priority topic of great interest in the dental field. As a result of our work, we presented the following seminars at the WHO Seminars "Oral Health in Transition": *Oral health in pregnant women*; prof. dr. Marie Janne Aldea, lecturer Sorina Solomon, associate professor Dr. Mărioara Păvăleanu; *Oral health of AIDS patients*; prof. dr. V.Luca, associate professor dr. Carmen Dorobăţ, lecturer dr. Sorina Solomon, Dr. Skowronski Anton
- „Evaluation of malnutrition-inflammation syndrome and its influence on survival in patients with chronic end-stage renal disease treated by dialysis", Internal Grant UMF nr. 20432/ 27.09.2013 that I participated to as a member.
- „Assessment of root cementum surface corresponding periodontal pockets after instrumentation with: Gracey curette, piezoelectric ultrasonic device and Periotor reciprocating instruments", Internal Grant UMF nr.30879/30.12.2014 that I have directed.

The overall postdoctoral research efforts have generated the following publishing outcomes: *21 artic*les published as main author in ISI publications, FI>0,3; *15 articles* published as collaborator in ISI publications, FI>0,3; *35 articles* published as main author in IDB/B+ journals; *43 articles* published as collaborator in IDB/B+ journals.

INTERNATIONAL SCIENTIFIC VISIBILITY

- HIRSCH INDEX(CLARIVATE ANALYTICS): 14
- NUMBER OF PUBLICATIONS IN CLARIVATE ANALYTICS DATABASE: 58
- CUMULATIVE IMPACT FACTOR (main author): 29,017
- TOTAL NUMBER OF CITATIONS WITHOUT SELF-CITATIONS(CLARIVATE ANALYTICS): 249
- AVERAGE CITATION PER ITEM: 7,14
- HIRSCH INDEX (GOOGLE SCHOLAR): 11
- ORCID: https://orcid.org/0000-0002-9588-6910

CHAPTER I

MODERN DIAGNOSTIC METHODS FOR PERIODONTAL DISEASE

I.1 State of the art in oral fluids periodontal diagnosis

For oral care clinicians, there may be a degree of uncertainty in estimating the outcomes and stability of specific successful periodontal treatment. When using conventional probing depth approach as a measure of disease progression, more than 2 mm of clinical attachment loss must occur before a site is considered to be "advanced", making this monitoring method an assessment of the "history" of the disease rather than a real-time assessment of the disease's activity (Lindhe et al., 2015). Thus, an innovative diagnostic tool is needed, with the ability to detect real-time periodontal changes.

Oral fluids, such as gingival crevicular fluid and saliva, have emerged as additional diagnostic tools. Having in mind that oral fluids are easy to collect and they also contain local and systemic biomarkers, they may offer the basis for patient-specific diagnostic tests for periodontal disease.

Saliva is a liquid, rich in serum albumin and antimicrobial and immunomodulatory proteins, which contributes to the lubrication of the mucosa, the protection of the dental structure and the integrity of the oral cavity. Studies have revealed the potential to identify and measure panels of saliva biomarkers for diagnosing periodontal disease and monitoring progression and health (Korte & Kinney, 2016). The physiology of periodontal tissues and periodontal diseases are very complex and have provided challenges in reaching the goal of salivary diagnosis for individualized treatment and maintaining periodontal health.

Many studies have been published that have supported the idea that periodontal disease can really influence a number of systemic diseases, such as diabetes, cardiovascular events and osteoporosis. One hypothesis is that bacteria in the plaque biofilm enter the bloodstream and cause infections and inflammation in a distant site. Another relates to the stimulation and release of pro-inflammatory cytokines or acute phase proteins to a distant site that may intensify or initiate a disease process. Further research is needed to clarify these associations, but the links are explored and may provide a more detailed explanation of oral-systemic links (Genco & Borgnakke, 2013; Sima & Glogauer, 2013). The role of inflammation seems to be a common denominator between periodontal diseases and some systemic diseases, which underlines the importance of using salivary diagnosis for periodontal diseases and monitoring their evolution.

Current clinical assessments used to determine the severity of periodontal disease, response to therapy, and disease activity include: depth of the periodontal pocket; the level of clinical attachment; bleeding on probing; gingival inflammation; presence of bacterial plaque or level of oral hygiene care; radiographically detectable bone suppuration and bone loss.

Clinical evaluations are an important element of the periodontal disease diagnosis, but can offer information only after the biological onset of the disease process. In addition, measurement errors, such as probe angulation and force, may interfere with precise measurements of attachment level and may be significant enough to distort the clinical treatment plan. These clinical measurements are useful for evaluation, but they alone are not able to determine the actual activity of the disease or the future risk of periodontal tissues loss.

Saliva is mainly produced by the three major pairs of salivary glands (parotid, submandibular and sublingual), with smaller amounts of saliva coming from hundreds of minor glands located in the oral, vestibular and palatal tissues. Saliva also contains non-

salivary elements, such as gingival crevicular fluid, squamous cells, nasopharyngeal discharges, external debris, as well as bacteria and bacterial by-products (Roblegg et al., 2019). The typical saliva flow varies between 800 and 1,500 ml / day, which makes it a readily available biological fluid.

Another advantage of using saliva as a "real-time" diagnostic sample is that it can be collected in a comfortable way. Unlike the blood collection and fear associated with the needle, or a urine sample and the intrusion of privacy, saliva can be collected in a non-invasive manner. Deviations of salivary and flow production can be influenced by factors such as time of day, duration of collection time, temperature, hydration status of the patient, systemic health status or emotional state (Korte & Kinney, 2016). Despite these potential limitations, the analysis of saliva biomarkers has been shown to detect, monitor and comply with treatment recommendations in both general and dental medicine.

When the periodontal pathogenic processes are considered, the periodontitis can generally be divided into three phases: inflammation, degradation of the connective tissue and bone turnover. During each phase of the disease, host-specific biomarkers were identified and, therefore, which provide a general sense of the stage of pathological tissue decomposition. In the early inflammatory stage of the disease, numerous cytokines, such as prostaglandin E2, interleukin-1, interleukin-6, and tumour necrosis factor alpha, are released from a variety of cells, such as those of the junctional epithelium, fibroblasts, connective tissue, macrophages and polymorphonuclear neutrophils.

As the disease progresses, strong enzymes, such as matrix metalloproteinase-8, matrix metalloproteinase-9 and matrix metalloproteinase-13, are released to the infected site, leading to the destruction of connective tissue collagen and alveolar bone loss. As the disease becomes more severe, levels of tumour necrosis factor, interleukin-1 and RANKL are increased, and ultimately mediate osteoclastogenesis and alveolar bone destruction (Korte & Kinney, 2016).

Although individual biomarkers have been studied as indicators of the development of periodontal disease, it is unlikely that an autonomous biomarker will present with a sufficiently high level of specificity and sensitivity to meet the criteria of a diagnostic tool. However, evidence indicates pathogens that may offer promising applications for differential diagnosis, treatment planning and monitoring, and for identifying patients at risk for future tissue destruction (Ghallab et al., 2018).

Combined salivary markers are a superior tool in predicting the progression and stability of periodontal disease. One study examined six salivary protein biomarkers - matrix metalloproteinase-8, osteoprotegerin, inflammatory macrophage protein-1alfa, interleukin-1beta, interleukin-8, and tumour necrosis factor - associated with chronic periodontitis (Sexton et al., 2011). These biomarkers are involved in inflammation, degradation of connective tissue and turnover of alveolar bone modulated by osteoclasts. This study demonstrated that three of the proteins - macrophage inflammatory protein-1a, matrix metalloproteinase-8 and osteoprotegerin - are potentially useful in monitoring periodontal disease. In addition, matrix metalloproteinase-8 was the best biomarker of response to therapy. Similarly, Kinney et al. (2011) demonstrated a group of markers (respectively matrix metalloproteinase-8, matrix metalloproteinase-9, osteoprotegerin and interleukin-l beta) that, at low concentrations, predicted periodontal stability in longitudinally monitored subjects.

Other combinations of biomarkers that have been identified together in significantly higher numbers in subjects with periodontal disease compared to healthy control subjects are matrix metalloproteinase-8, tissue inhibitor metalloproteinase-1, and collagen type I telopeptide (Yee et al., 2017). In another cross-sectional investigation, matrix metalloproteinase-8 and interleukin-1-beta were found to be significantly higher in subjects with periodontitis compared to healthy control subjects (Kuboniwa et al., 2016). In addition,

these two markers demonstrated a positive correlation with periodontal indices, bleeding on probing, clinical attachment level and percentage of sites with probing depth higher than 4 mm.

Gingival crevicular fluid (GCF) is both a physiological fluid and also an inflammatory exudate that originates from the vessels of the gingival plexus of blood vessels and flows through the external basement membrane and the junctional epithelium to reach the gingival sulcus. The presence of fluid in the gingival sulcus has been described since the 19th century (Barros et al., 2016).

It has been demonstrated that gingival fluid can be isolated from a healthy sulcus, although only in small amounts. However, leukocyte infiltrates can be seen throughout the junctional epithelium, and PMN leukocytes can always be found in the sulcus, even in clinically healthy situations in which the flow of gingival crevicular fluid is relatively low (Roblegg et al., 2019).

Various inflammatory molecules are released from cells of the sulcular and junctional epithelia, dendritic cells, fibroblasts, macrophages and neutrophils. Moreover, various enzymes, such as matrix metalloproteinases (MMP), are produced by neutrophils, fibroblasts and osteoclasts, leading to the degradation of connective tissue collagen and alveolar bone. More than 90 different components in gingival crevicular fluid have been evaluated for periodontal diagnosis.

Collection of GCF is a simple, non-invasive manoeuvre; therefore, this analysis has been extensively explored in the quest for potential diagnostic biomarkers of periodontal disease. There are various methods of GCF sampling, that most often include collection of gingival crevicular fluid on paper strips to measure specific components. Most studies collect gingival crevicular fluid using standardized strips/paper points of filter paper (Periopaper).

Researchers continuously identify and test the complex "signatures" of periodontal disease, which will allow accurate diagnosis and improvement of individualized care. New technologies are available that are capable of measuring salivary biomarker panels for accurate diagnosis and treatment recommendations for periodontal disease.

Because specific biomarkers for periodontal disease and evolution are determined by longitudinal analysis, it appears that the technology is ready to cope with scientific discovery. Both will come together to enable healthcare providers to improve the prevention and treatment of periodontal disease through personalized treatment.

Personalized treatment is a medical model that uses genetic, genomic, environmental and clinical diagnostic tests to individualize patient care (Giannobile, 2012). This approach uses clinical assessments and subclinical profiles to develop highly individualized diagnostic, prognostic and treatment algorithms. The use of this model in oral health care, especially in periodontology, has the potential to provide discriminatory patient stratification models to improve personalized treatment algorithms.

Personalized treatment for periodontal disease may involve the use of saliva and gingival crevicular fluid to develop subclinical profiles, identify and measure specific genotypes, phenotypes, pathogens, inflammatory markers and biomarkers of collagen degradation, to make clinical decisions aware of disease sensitivity, specific risks and treatment interventions.

When considering the possible use of saliva and GCF in a customized model for periodontal disease, we can imagine the implementation on several levels - the detection of the disease, the monitoring of the treatment results and the identification of the refractory or progressing cases.

In the screening phase, the use of saliva and GCF to identify patients at risk for future disease activity provides the opportunity for increased risk management strategies, preventive care and / or behaviour change on the part of the patient to prevent the onset of the disease.

In the diagnostic stage, the identification of the early stage of the disease can allow a less invasive, less expensive treatment. Saliva and GCF, as a simple mechanism for monitoring treatment outcomes, as well as identifying refractory sites, also provide valuable information to the patient and clinician regarding the current state of the disease.

The formation of biofilms in different environments, including clinical situations, has been studied intensively using a great variety of microscopic techniques. SEM is a precious tool for ultrastructural investigation of the general aspect of the biofilm and its characteristics: bacterial species, individual bacterial cells, the glycocalyx and the presence of inorganic biofilm components. There are various descriptions of SEM use in biofilm assessment on implants, prosthetic devices, catheter, teeth or other solid structures in order to establish the role of biofilms in the persistence of infections. The conventional SEM technique needs a complicated procedure: sample fixation in glutaraldehyde and/or in osmium tetroxide, followed by dehydration and coverage ('sputtering') of the biofilm with conductive metallic material (Gold, Palladium) or Carbon. The low-vacuum SEM observation method differs from traditional preparation protocols for SEM examination; the method is simple, quick and offers sample protection.

I.2 Saliva as a source for DNA extraction methods in PCR - Amplifiable Genomic DNA

This research direction has been materialized by publishing the following paper:
Solomon S, Badescu AC, Jelihovschi I, Iancu LS, Teusan A, Martu S. Evaluation of DNA extraction methods from saliva as a source of PCR-amplifiable genomic DNA. Rev. Chim (Bucharest) 2015; 66(12): 2101-2103.
http://www.revistadechimie.ro/pdf/SOLOMON%20S.%2012%2015.pdf

Aim of the study

We conducted a study with the aim to evaluate the DNA yield and the suitability of isolated DNA for real-time PCR amplification (which is the most used molecular technique in genetic studies) using an automatic nucleic acids extractor, a commercial DNA extraction kit and a simple cost effective protocol with ammonium acetate under different storage conditions of saliva samples.

Materials and method

The study was performed on a group of 20 volunteers of both genders (age range: 19-57). The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study. The study subjects were advised not to brush their teeth, to smoke, to eat or drink at least 2 h prior sample collection. Each individual was asked to provide 10 mL of whole saliva and was oriented to rub the tongue vigorously on the teeth and whole oral mucosa surface.

In order the sample to be more representative and to avoid sub-sampling errors the participants were also asked to rinse vigorously their mouth for 45-60 s with 15 mL of a 2% saline solution. Collection of the oral rinse was done in the same 50 mL Falcon tube with the priory collected saliva with an interval of 5 min between the two different sample collections.

In order to assess the DNA integrity over time the samples were divided into 4 tubes containing equal amount of sample and were submitted to different storage extraction (C1), the second tube was stored at -20^{o}C for 1 week (C2), the third tube was immediately centrifuged at 10000 rpm for 5 minutes and the cell pellet stored at -20^{o}C for 1 week (C3) and the final aliquot was mixed with ethanol to a final 70% concentration and stored at room temperature for 1 week (C4). A total number of 20 samples was included for the study, 5

samples for each storage conditions category [5 x (C1+C2+C3+C4)].

Genomic DNA was extracted in three different ways:

a) using an automated nucleic acids extractor, MagCore® Super (RBC Bioscience Corp., Taiwan) with the MagCore®Genomic DNA Tissue Kit, cartridge code 401 following manufacturer's instructions (E1);

b) QIAamp® DNA Mini Kit (QIAGEN Group), spin protocol following manufacturer's instructions (E2),

c) Following the modified Aidar and Line protocol reported by Kuchler et all with minor modifications (E3) (Aidar & Line, 2007; Kuchler et al., 2012).

Briefly we pelleted the buccal cells by centrifugation at 10000 rpm for 5 min and washed the pellet 2 times with PBS and 1 time with molecular biology pure water by centrifugation at 3500 rpm for 3 min and the supernatant was discarded. The pellet was transferred to 2 mL microcentrifuge tube and a 1 mL of cell lysis solution [10mM Tris, pH 8.0, 5mM EDTA, 5mM sucrose, 0.5% SDS] containing proteinase K (150 ng/mL) was added and incubated for 5 h at 57^0C. After the incubation the proteins and other contaminants were removed by adding 400 µL of 10mM ammonium acetate followed by vigorous vortexing for 15 s and centrifuging at 17000 x g for 10 min.

The supernatant was transferred to a new tube to which 800µL of isopropanol was added and DNA was precipitated at -20^0C for 30 min. After a centrifugation step at the supernatant was poured of followed by a washing step with 1mL of 70% ethanol and centrifugation at17000 g for 10 min at 4^0C, the supernatant was discarded, the tube was inverted and air-dried for 30 min on absorbent paper. And finally the DNA was re-suspended in 150 µL of TE buffer [10mM Tris (pH 7.8) and 1mM EDTA]. A total number of 60 DNA extractions were performed (each sample was extracted by the 3 different methodologies, E1, E2 and E3).

All the chemicals used in this protocol were molecular biology grade and were acquired from Sigma Inc.

The total DNA yield and purity was determined by spectrophotometry. We used the incorporated spectrophotometer in the MagCore® Super automated nucleic acids extractor (RBC Bioscience Corp., Taiwan). The DNA concentration is obtained by readings of optical density (OD) at 260 nm and the ratio of ODs at 260nm/280nm is used to estimate the DNA purity. In general, a 1.7-2.0 value of ODs 260nm/280nm ratio is indicative for acceptable DNA purity.

In order to assess the DNA quality of samples after different storage conditions and different DNA isolation methodologies in terms of their real-time PCR amplification efficiency the samples were submitted to a PCR reaction targeting a 268 bp fragment of human beta-globin gene.

Real-time PCR reactions were performed on a DT prime, plate type real-time cycler (DNA-technology, Russia) using a commercial human beta-globin control assay (PC04 and GH20 primer set, Life Technologies), following the manufacturer's instructions. This internal control assay contains all the reagents necessary for amplification and quantification of a 268 bp fragment of human beta-globin gene.

Results

The total DNA yield was generally consistent across different storage conditions, except the C4 conditions where an important decrease in the amount of DNA regardless the extraction methodology was observed (Table I.1).

Also the real-time PCR CT value was slightly modified for the C4 samples but the difference didn't have a negative impact on final amplification of the target proving that even in such conditions we can obtain real-time PCR amplifiable human genomic DNA from

saliva. Obtaining PCR amplifiable DNA from saliva can be done at reduced costs, according to the ammonium acetate DNA extraction protocol (E3), and in this way we can scale-up the studies in order to obtain more reliable data.

The results obtained by E3 protocol comparable with the others protocols used in the study (E1 and E2), doesn't use toxic reagents (phenol or chloroform), several samples can be processed in parallel and obviously implies much lower costs.

Table I.1. Effect of storage conditions and DNA extraction protocols on yield, purity and quality of genomic DNA from saliva. Data provided as mean values and (range)

Storage conditions	DNA extraction method		Total DNA yeld (μg)	DNA purity $OD_{260/240}$	DNA quality real-time PCR C_r value
C1 Immediate extraction	E1	Automated extractor	32.83 (23.11-53.42)	1.82 (1.7-1.9)	23.57 (23.27-23.84)
	E2	QIAmp DNA extractor mini	28.31 (22.88-55.45)	1.87 (1.73-1.94)	
	E3	Ammonium acetate protocol	27.88 (15.00-52.33)	1.97 (1.7-2.01)	
C2 Immediately stored at -20^{0}C for 1 week	E1	Automated extractor	32.35 (22.47-50.92)	1.87 (1.74-1.92)	23.68 (23.34-23.82)
	E2	QIAmp DNA extractor mini	29.42 (21.24-54.83)	1.85 (1.7-1.95)	
	E3	Ammonium acetate protocol	25.62 (18.39-46.28)	1.95 (1.65-2.00)	
C3 The pellet stored at -20^{0}C for 1 week	E1	Automated extractor	35.45 (22.12-48.32)	1.85 (1.72-1.94)	23.65 (23.29-23.81)
	E2	QIAmp DNA extractor mini	30.84 (19.32-49.34)	1.89 (1.75-1.9)	
	E3	Ammonium acetate protocol	27.49 (17.56-48.57)	1.97 (1.65-2.02)	
C4 70% ethanol at room temperature for 1 week	E1	Automated extractor	7.28 (1.85-15.42)	1.92 (1.76-1.98)	26.44 (26.02-26.94)
	E2	QIAmp DNA extractor mini	8.7 (2.37-10.78)	1.87 (1.72-1.95)	
	E3	Ammonium acetate protocol	6.56 (1.53-8.34)	2.0 (1.62-2.02)	

Discussion

The reason for the decrease of DNA yield in C4 samples may reflect technical problems with these extractions and also may be due to the different sampling methods and sample amount submitted to DNA isolation in comparison with Kuchler et all. (2012) and Aidar and Line (2007) studies.

The use of an automatic nucleic acids extractor (E1) is generally more advantageous over the commercial DNA extraction methods (E2). Using such technologies, the hands-on time is drastically reduced, and the risks of cross-contamination of samples, nucleic acids contamination of the laboratory facility are also reduced. The costs between E1 and E2 protocol are comparable as long as E1 protocol needs less additional materials and is suitable for high-throughput pipelines.

Conclusions

Saliva is a viable alternative source for real-time PCR amplifiable DNA and represents a flexible body fluid to work with. Even storage in 70% ethanol solution for one week at room temperature, still provided DNA sufficient for several real-time PCR reactions without affecting the PCR amplification results.

We conclude that collection, preservation and isolation of DNA from saliva can be done at low costs and provides flexibility for the clinical and laboratory workflow.

I.3 The importance of gingival crevicular fluid in the assessment of bacterial associations involved in periodontal disease

This research direction has been materialized by publishing the following paper:
Teodorescu AC, Teslaru S, Solomon SM*, Zetu L, Luchian I, Sioustis IA, Martu MA, Vasiliu B, Martu S. Assessment of bacterial associations involved in periodontal disease using crevicular fluid. REV. CHIM (Bucharest) 2019; 70(6):2145-2149**.**
https://revistadechimie.ro/pdf/53%20TEODORESCU%206%2019.pdf

Aim of the study

The aim of the study was to assess the bacterial subgingival profiles involved in chronic and aggressive periodontal disease using crevicular fluid and to highlight the possible bacterial associations that are characteristic to each clinical form, thus making the differential diagnosis easier for the general dental practitioner.

Materials and method

The study was conducted on 54 patients, between October 2016 and January 2018. These patients were examined at the Periodontal Department of the Dental Medicine Faculty of ”Grigore T. Popa” University of Medicine and Pharmacy from Iasi and in a private practice in the same city.

For the patients to be included in this study, they had to have the ages between 18 and 58 years old and to present a form of periodontitis, be it generalised aggressive or severe chronic. The exclusion criteria consisted in: periodontal therapy 6 months prior to the beginning of the study and systemic antibiotic therapy 3 months before, pregnant or breastfeeding women, important systemic diseases such as leukaemia, malignant tumours, recent acute cardiac episodes, anticoagulant therapy, ongoing bisphosphonates therapy or in the 12 months prior to the beginning of the study. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

After a thorough periodontal clinical exam, the patients were divided into 2 groups: the GAP and the SCP. For each patient we selected 5 teeth, the 5 most affected sites (the deepest periodontal pockets or the highest attachment loss). One at a time, each site was isolated with sterile cotton rolls and the saliva was removed the surface of the teeth with the help of air spray. Using sterile paper points, crevicular fluid filled with bacteria was collected from each selected periodontal pocket. For this stage we used the PET kits made by MIP Pharma GmbH. The paper points were left in place for 20 s and then put into a transport Eppendorf tube and shipped to the laboratory in Germany. There, the crevicular fluid samples were analysed as cumulated sample through a Real-Time PCR technique (polymerase chain reaction).

The working principle for the Real-Time PCR consists in DNA amplification and quantification of the PCR product in one stage through fluorescence. Thus, fluorescence is proportional with a number of DNA amplifications. The applications of Real-Time-PCR in medicine and biology are the detection and quantification of bacterial and viral pathogens, genotyping, the quantification of genic expression and the analysis of DNA deterioration. The manufacturer presents numerous advantages for this type of Real-Time-PCR testing: accurate quantification of periodontal pathogens, high reproducibility, high specificity, high sensitivity, quick detection (short cycles of 1-1,5 hours) and low risk of contamination.

The PET Plus test shows the presence and quantification of 9 periodontal pathogens in each examined sample: *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Treponema denticola* (Td), *Tannerella forsythia* (Tf), *Prevotella intermedia* (Pi), *Peptostreptoccocus micros* (Pm), *Fusobacterium nucealtum* (Fn), *Eubacterium nodatum* (En) and *Capnocytophaga gingivalis* (Cg).

Results

The study was performed on 54 patients, 29 males and 25 females, from which 29 with GAP and 25 with SPC. The ages of the study participants ranged between 20 and 56 years, with a mean value of 38.78 ± 9.189 years.

In order to compare the age mean values between the two study groups we used the t-test. It is obvious that the mean age value was significantly lower for the GAP patients (35.31±1.468 years) than the mean value for the SCP group (42.8±1.813 years) with a value of p=0.02.

For the SCP study group, 4 of the 9 periodontal pathogens had a very high prevalence of over 90%: Pg, Td, Pm and Cg. The other 5 bacteria were present but with lower prevalences: Aa 16%, Fn 32% and Ee 44%. When we analysed the GAP group we observed that Td was present in 100% of the patients and other 4 pathogens were highly prevalent (over 90%): Pg, Tf, Pm and Cg. The only bacteria with a lower prevalence (under 50%) were Aa.

When we compared the two study groups it was clear that Aa, a bacteria correlated usually with aggressive periodontitis forms, was not only present in the GAP group (11 patients) but also in 4 cases of SCP, without a statistically significant difference. More so, all of the 9 periodontal pathogens showed a higher prevalence for the GAP group, significantly higher for Fn (p=0.007) and En (p=0.007).

A second step was analysing the quantities of each periodontal pathogen. We noticed that for the SCP group the bacteria with higher mean values were Td (7.08x105±1.06x106), Tf (5.33x105±1.16x106) and Pg (4.35x105±5.35x105) and with lower mean values were Aa (2.59x103±2.89x103) and En (3.02x104±5.92x104).

For the GAP study group, we found that the bacteria with higher mean counts were Pg (7.34x105±8.86x105), Pi (6.65x105±8.23x105) and Td (5.56x105±5.87x105), as for the bacteria with lower mean counts we found En (9.3x103±1.15x104) and Aa (8.57x104±8.25x104) (Figure I.1).

We used the Mann-Whitney non parametric test to highlight the possible differences between the two study groups and it was obvious that there were statistically significant differences, the GAP patients exceeded higher quantities of Aa and Pg.

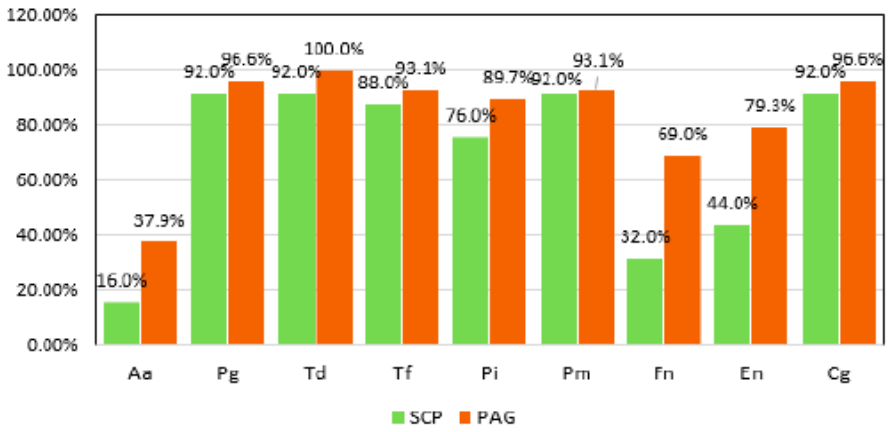


Figure I.1. The compared prevalence of the 9 periodontal pathogens for the 2 study groups

Discussion

The mean age for the GAP patients was significantly lower (35.31±7.9 years) than for the SCP patients (42.8±9 years) concordantly with the literature data that aggressive periodontitis has its onset early in life, before the age of 25 (Albandar, 2014), and so the diagnosis is also made earlier comparing to the chronic periodontitis cases that are usually visible after the age of 35.

From a pathogen point of view, the prevalence of Aa was much higher in the GAP group (11 patients) compared to the SCP group (4 patients), without having a statistically significance. Our results were similar to those of another study, made on 260 patients of which 75 with GAP and 185 with CP, that found no significant differences concerning the prevalence of Aa, but without analysing the quantity of bacteria in the collected samples (Rosenstein et al., 2004). Other studies along the way correlated the presence of this periodontal pathogen with either aggressive periodontitis (Chahboun et al., 2015) or with chronic forms (Cardoso et al., 2018).

P. gingivalis had a high prevalence in both of the study groups (92% in SCP versus 96,6% in GAP) without a statistical difference between the two. Other studies reported similar results, saying that Pg is not a bacterium with a high differential diagnostic ability, because it can be found in both forms of periodontitis (Tomita et al., 2013). More so, Pg could be an opportunistic pathogen that causes periodontal disease only in favourable conditions (Griffen et al., 2012). Heller et al. (2012) compared the microbial profiles of GAP and CP (chronic periodontitis) and found a significant correlation between the prevalence of Pg and the chronic periodontitis group. Contrary to these findings, other studies have found that higher quantities of Pg are significantly correlated with cases of aggressive periodontitis (Oettinger-Barak et al., 2014; Chahboun et al., 2015). The same correlation was found in the present study where higher counts of Aa and Pg were found in the GAP group.

In the present study we found significant differences in the prevalence of Fn and En, which were correlated with the GAP group. These results are partially sustained by our previous preliminary study, made on 20 patients, that found a qualitative and quantitative correlation of Fn with the GAP group (Teodorescu et al., 2017).

Fn is a Gram negative, anaerobe microorganism with a good adhesion capacity within the oral biofilm, that has got a lot of attention from medical specialists and researchers because he is believed to be an opportunistic commensal, not only for the periodontal disease (Han, 2015). It is one of the most frequent bacterial species in the oral cavity of healthy and periodontal impaired patients, be it a simple form of gingivitis or a chronic or aggressive periodontitis (Griffen et al., 2012; Feng et al., 2015).

Fn plays an important role in the formation and maturation of dental biofilm and it is the microorganism that ties the commensals that attach initially and the pathogens that are later colonisers in the dental biofilm.

Additionally, there are researchers who found that Fn is capable of sustaining the growth of Pg in oxygen filled and CO_2 lacking environments, where Pg would not be able to normally survive (Krupa et al., 2018). The results of our study confirm this idea, considering the fact that the GAP group showed a significantly higher prevalence of Fn and higher quantities of Pg, suggesting that there is a strong correlation between the two periodontal pathogens.

Concerning the significantly higher presence of En in the GAP group reported in our study, it comes to support the conclusions of other studies that found a strong correlation between these bacteria and aggressive forms of periodontal disease (Elabdeen et al., 2014). En is an asaccharolytic, anaerobic and nonreactive bacterium that grows with great difficulty on culture environments (Arora et al., 2014), but which seems to become more and more abundant once the aggressive periodontitis patient grows older.

Taking into account the mixt results shown in the recent studies and their limited numbers comparing subgingival microbial profiles of aggressive and chronic periodontitis, Real-Time PCR testing can be very useful, using either crevicular fluid or saliva. This type of analysis will uncover the periodontitis cases, chronic or aggressive, that harbour bacteria such as Aa and Pg. The presence of either of the two periodontal pathogens in high numbers, above the pathogenic threshold, justifies the use of systemic antibiotics during MINST (minimal invasive non-surgical therapy). Using this type of testing for patients with important periodontal damage, periodontists and general dentists will be able to recommend a specific antibiotic therapy, and not an empirical one, thus limiting the global antibiotics usage and lowering the risk of bacterial resistances.

Our study has its own limitations. It has been made on a small group of 54 Caucasian patients. The general prevalence of aggressive periodontitis is pretty low, affecting more the African-Americans. It is believed that its prevalence varies from 0,1% in Central and Northern Europe, to 2,6% for the black population in Northern America and to 1-5% for the African populations (Susin et al., 2014). Unfortunately, there are no available data regarding the prevalence of chronic or aggressive periodontitis for the Romanian population. For the enrolment in this study, one of the exclusion criteria was a systemic antibiotic therapy 3 months prior to the beginning of the study. This limitation reduced furthermore the number of patients available to participate in such a study because, in Romania, a lot of people take frequently as self-medication antibiotics for different types of systemic diseases, colds and flues.

Another limitation for our study was the use of the commercial test PET Plus from MIP Pharma GmbH, a test which determines the presence and numbers of only 9 periodontal pathogens. More studies are necessary in order to highlight the presence of other periodontal pathogens implicated in chronic and aggressive periodontitis, studies which should be made on larger study groups. These will make possible the discovery of new periodontal bacteria with a key role in differential diagnosis and more precise results.

Conclusions

There is a correlation between the presence of two periodontal pathogens, Fn and En, and a PAG diagnosis. Additionally, the quantitative evaluation of the 9 periodontal pathogens has shown higher number of bacteria for PAG patients compared to SCP patients. Aa and Pg, the main periodontal pathogens, had significantly higher values in the PAG group. The individualised determination of periodontal pathogens, using any method available and especially RT-PCR, helps steering a systemic antibiotic therapy and limits the self-medication and the possibility of developing bacterial resistances.

I.4 The diagnosis of endo-periodontal lesions

State of the art in the endo-periodontal lesions ethiopathogeny

Inflammation and resorption of the alveolar bone in most cases is a consequence of the interaction between the microbial infection and the response of the host (Buduneli, 2021). The critical role of bacteria in the development of periapical lesions was demonstrated by mechanical exposure of dental pulp to the oral cavity of germ-free animals. In these animals, the pulp exposure is healed with an initial or transient inflammatory response in pulp tissue, followed by a reparative response from the pulp cells and leading to the formation of a new dentin-like matrix linking to the exposed site. In contrast, mechanical pulp exposure in animals with normal oral bacteria causes an infection of the dental pulp, pulp tissue necrosis and chronic infection that prevents the repair process. The infection persists because necrotic

tissue of dental pulp is inaccessible to leukocytes and therefore constitutes a bacteria-protected reservoir (Graves et al., 2016). Chronic inflammation stimulated by bacteria and their products in the periapical area of the tooth leads to localized bone resorption, which is "decoupled", so there is no bone repair without treatment. The result consists in the formation and expansion of granulomas or cysts in apical tissues (Zhang et al., 2018).

Periodontium consists in a set of tissues in the immediate vicinity of the tooth, with a complex biofilm that includes various bacterial species (Curtis et al., 2020). Although the consensus is that periodontal diseases are stimulated by bacterial adhesion to the surface of the teeth, there is controversy over which bacteria stimulate the irreversible degradation of periodontal tissues in periodontitis. The presence of microbial pathogens in periodontal and periapical environments triggers an initial production of proinflammatory cytokines such as TNF-α and IL1-β that stimulate the expression and activation of matrix metalloproteinases (MMPs) that degrade the extracellular connective tissue matrix (Graves et al., 2011). Cytokines such as TNF-α can stimulate osteoclastogenesis independently, while other cytokines stimulate RANKL expression which leads to the formation and activity of osteoclasts (Ono & Nakashima, 2018). Innate and acquired combined immune responses can lead to high levels of inflammation and bone resorption (Hirao et al., 2009). These proinflammatory cytokines are believed to generate an amplification loop that contributes to the progression of periodontal and periapical lesions. On the contrary, cytokines produced by Th2 and Treg cells, such as IL-4 and IL-10, have the opposite effect in part by stimulating the production of matrix metalloproteinase and OPG inhibitors, as well as by limiting cytokine production inflammatory.

This research direction has been materialized by publishing the following papers:

1.Zaharescu A, Solomon SM*, Luca MG, Toma V, Luchian I, Sufaru IG, Martu MA, Foia L, Martu S**.** Quantification of proinflammatory molecules (IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α) in crevicular fluid and serum in patients with endo-periodontal lesions. Rev. Chim (Bucharest) 2019; 70(6):2252-2255**.**

https://revistadechimie.ro/pdf/76%20ZAHARESCU%206%2019.pdf

2.Rusu D, Stratul SI, Calniceanu H, Boariu M, Ogodescu A, Milicescu S, Didilescu A, Roman A, Surlin P, Locovei C, Chiperi M, Solomon S*, Nica L. A qualitative and semiquantitative SEM study of the morphology of the biofilm on root surfaces of human teeth with endodontic-periodontal lesions. Exp her Med. 2020; 20(6): 201.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7593830/

I.4.1 The inflammatory molecular diagnosis

Aim of the study

We conducted a research which proposed an assessment of the localized inflammatory burden but also at the systemic level by quantitating the pro-inflammatory molecules (IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α) in subjects with endo-periodontal lesions.

Materials and method

This clinical and paraclinical study was performed on a group of 146 subjects; the mean age was 46.28 ± 11.37 years. The group was comprised of 67 female subjects (45.89%) and 79 male subjects (54.11%). As far as the environment of origin is concerned, 53 subjects came from rural areas (36.30%) and 93 from urban areas (63.70%).

The subjects, following clinical and radiological examinations, were divided into five groups: healthy endo-periodontal patients (n=24) (group I), patients with superficial periodontitis (probing depth less than 4mm) (n=36) (group II), patients with moderate periodontitis (probing depth of 4-6mm) (n=32) (group III), patients with severe periodontitis

(probing depth greater than 6mm) (n=25) (group IV) and patients presenting endo-periodontal combined lesions (n=29) (group V). Patients in groups II, III and IV did not have active endodontic lesions. Also, all subjects included in the study were systemically healthy.

We excluded patients with anti-inflammatory medication over the past 6 months, patients who had endodontic or periodontal treatment in the last 12 months and smokers.

The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

The patients underwent a complex endodontic and periodontal clinical examination. Clinical examinations were completed with retro-dental-alveolar radiographs.

For crevicular fluid analysis of IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α, the teeth were isolated with cotton rolls before harvesting crevicular fluid samples. The supra-gingival plaque was also carefully removed and the sites were gently dried with the air spray. A sterile paper cone was inserted into each selected periodontal site, left for 30 seconds, and then immediately inserted into sterile Eppendorf tubes which were stored at -20°C. In the case of visible contamination with blood, the paper cone has been removed and a new site has been selected. For the determination of inflammatory markers, the paper cones were thawed, cut at 1 cm in length and thawed with 50 µl 1X [13 mM Na2HPO4, 7 mM NaHPO4, 100 mM NaCl (pH 7.0)] phosphate buffer at 4°C. Further, the paper cones were centrifuged at 13000 xg for 10 minutes at 4°C (Sufaru et al., 2016).

For serum assay of IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α, a 15 ml blood sample was obtained by venipuncture from each participant. The serum was collected by centrifugation, aliquoted, stored and processed at the end of the study. Inflammatory markers were identified simultaneously using flow cytometer multiplex assays (BD ™ Cytometric Bead Array (CBA, BD Bioscience, San Jose, CA, USA). CBA is a series of discrete spectral particles that can be used to capture soluble analytes. Analyses are then measured by fluorescence emission and flow cytometry detection. The method uses a series of different particles that are stably labelled with a fluorescent dye whose emission wavelength is read at ~ 650 nm. Each different group is labelled with a discrete level of fluorescent dye so that it can be distinguished by mean fluorescence intensity. The spheres in each group are covalently coupled to antibodies that can specifically capture a particular type of molecule present in biological fluids. This type of test was chosen because it allows a full analysis of the biomarkers involved in periodontitis and systemic diseases.

Results

IL1-α showed serum and crevicular fluid values significantly higher than the healthy subjects; also the values recorded in the group with endo-periodontal syndrome patients were significantly higher than the group with severe periodontitis. Similar differences were also observed for serum and GCF values of IL1-β.

Values for IL2, IL12, TNF-α and IFN-γ measured in crevicular fluid were higher for groups II, III, IV and V than the group of healthy endo-periodontal subjects; following comparison of the values obtained for groups IV and V, we noticed significant differences ($P < 0.001$) for the group with patients with endo-periodontal lesions. Values of proinflammatory molecules determined in crevicular fluid are shown in Table I.2.

IL2 showed significantly higher serum values for groups III, IV and V than group I; although the values recorded for the group with superficial periodontitis were higher, the differences did not reach a level of statistical significance ($P = 0.843$); it is noteworthy that the mean serum level of IL12 was significantly higher for the endo-periodontal syndrome group compared to subjects with severe periodontitis (Table I.2).

Serum IL12 values were significantly higher than healthy periodontal subjects only for patients with severe periodontitis and endo-periodontal syndrome, significantly higher for group V vs. group IV (P <0.001).

TNF-α and IFN-γ demonstrated significantly higher values for all pathological groups compared to the group of healthy endo-periodontal subjects; in addition, we noted for both proinflammatory molecules significantly higher serum values for the endo-periodontal syndrome subjects versus subjects with severe periodontitis. Data on serum determinations are shown in Table I.3.

Table I.2. Values obtained from crevicular fluid determination of proinflammatory molecules

GCF (pg/ml)	**I (n=24)**	**II (n=36)**	**III (n=32)**	**IV (n=25)**	**V (n=29)**	***P* Value group V/ group IV**
IL1-α	13.24±3.23	22.02±4.23	27.81±11.49*	33.45±8.68*	41.74±12.45*	*P* <0.05
IL1-β	12.32±5.81	19.21±7.91	25.76±10.66*	31.92±9.42*	43.19±12.97*	*P* <0.05
IL2	0.33±0.07	8.39±3.44*	29.97±11.84*	42.73±9.75*	56.59±21.32*	*P* <0.001
IL12	0.21±0.02	17.23±8.78*	21.48±7.32*	30.04±7.44*	46.94±22.64*	*P*<0.001
TNF-α	3.14±2.1	19.47±7.43*	28.77±7.37*	53.19±12.54*	79.83±27.97*	*P*<0.001
IFN-γ	2.88±1.14	11.29±5.22*	15.42±6.22*	24.32±10.03*	38.42±12.45*	*P* <0.05
Values are expressed as the mean value ± Standard Deviation; * indicates a p value of <0.05						

Table I.3. Values obtained from serum determination of proinflammatory molecules

Serum (pg/ml)	**I (n=24)**	**II (n=36)**	**III (n=32)**	**IV (n=25)**	**V (n=29)**	***P* Value group V/ group IV**
IL1-α	0.14±0.02	1.76±0.14	3.12±1.44*	4.33±2.38*	7.23±3.43*	*P* <0.05
IL1-β	0.31±0.12	1.44±1.26	4.22±2.37*	5.54±2.45*	8.41±3.59*	*P* <0.05
IL2	4.42±10.27	5.01±2.43	10.28±3.23*	19.21±7.33*	32.63±7.33*	*P*<0.001
IL12	1.21±0.09	1.97±1.28	2.76±1.49	5.29±2.98*	10.55±3.79*	*P*<0.001
TNF-α	0.65±0.10	3.02±1.12*	3.28±1.95*	6.84±2.55*	8.97±4.21*	*P*<0.05
IFN-γ	12.36±3.75	17.57±4.23*	19.24±6.44*	21.10±7.47*	38.94±9.79*	*P*<0.001
Values are expressed as the mean value ± Standard Deviation; * indicates a p value of <0.05						

Discussion

Endodontic lesions have been associated with multiple proinflammatory cytokines and chemokines. Proinflammatory molecules, especially IL1-α and IL1-β, are produced in peri-apical lesions by several cell types, including macrophages, osteoclasts, PMNs and fibroblasts. The role of IL-1 in stimulating periapical bone destruction has been demonstrated using interleukine-1 receptor antagonists to demonstrate a 60% reduction in lesion development (Siqueira & Rocas, 2014). It appears that much of the osteoclastogenic activity induced in periapical lesions is specifically related to interleukin-1a formation. However, when IL-1 receptor signalling is completely eliminated, there is an increase in the size of the lesion and systemic morbidity. In the present study, IL1-α showed significantly higher serum and crevicular fluid values than healthy subjects; also the values recorded in the group with patients with endo-periodontal syndrome were significantly higher than the lot with severe periodontitis, suggesting a much higher systemic load for these patients.

The IL2, IL12, TNF-α and IFN-γ levels measured in crevicular fluid were higher for groups II, III, IV and V vs. the group of healthy endo-periodontal subjects; After comparison of the values obtained for the groups IV and V we noticed significant differences (for the group with patients with endo-periodontal lesions.) TNF-α expression was identified in endodontic lesions by cells such as PMN, monocytes / macrophages and fibroblasts and may

contribute to lesion formation. In our study, TNF-α demonstrated significantly higher values for all groups with pathology than the group with healthy endo-periodontal subjects; In addition, we noticed significantly higher serum values for the endo-periodontal syndrome versus subjects with severe periodontitis.

IFN-γ is a lymphokine that has been implicated in periodontal bone loss. Mice with a genetic ablation of IFN-g have a greater *P. gingivalis*-induced bone loss compared to wild-type controls. T cells are an important source of IFN-γ in periodontitis and have been linked to the increase in RANKL expression (Ono & Nakashima, 2018). We obtained significantly higher serum levels of IFN-γ for all pathological groups compared to the group with healthy endo-periodontal subjects; moreover, the values were significantly higher for the endo-periodontal syndrome group versus subjects with severe periodontitis.

It is well known that, in its unsteady state, the spread of infection and the inflammatory process in the nearby tissue compartments is possible and can cause severe inflammatory conditions, but fortunately rare. Moreover, in view of increasing the awareness of a potential relationship between persistent, inflammatory oral cavity disorders and diseases of other organs of the body, acute and chronic manifestations of apical periodontitis can also be involved (Ostravik, 2019). Siqueira and Rocas (2014) cite how primordial apical periodontitis and post-treatment can influence the general health of the individual and remain a question that requires response in endodontic microbiology.

The possible link between chronic inflammatory processes of infectious origin and periodontal disease with systemic health is today one of the most interesting issues faced by the medical and dental scientific community. A question arises as to whether cell-to-cell direct interactions between periodontal or endodontic bacteria and host cells as well as between different human cells or autocrine and paracrine stimulation loops can influence the function of the distal tissues and organs resulting in pathogenesis or which contributes to the pathological mechanism of systemic diseases.

Conclusions

The values of inflammatory molecules in crevicular fluid of patients with periodontal pathology reflect the higher degree of local inflammation in endo-periodontal patients. Although patients with severe periodontitis and those with endo-periodontal syndrome exhibited the highest values, the levels were significantly higher for the latter, providing a much more severe molecular picture than the other patient categories.

Following serum determinations of proinflammatory molecules, patients with endo-periodontal lesions demonstrated significantly higher values even than subjects with severe periodontitis; these data indicate a much higher risk for these patients to develop and maintain systemic maladies, as is the role local inflammation can play over the general inflammatory status of the patient. Therefore, treatment at all stages of these patients acquires an increased level of complexity, higher even than cases of severe periodontitis.

I.4.2 The SEM analysis of the root surfaces biofilm of human teeth with endodontic-periodontal lesions

Aim of the study

We conducted a study with the aim to assess the biofilm on root surfaces of teeth with endo-periodontal lesions (EPL) with a modified protocol, using a simplified histological method to prepare specimens examined under low-vacuum SEM.

Materials and method

Using aseptic surgical techniques and sterile instruments, 25 teeth with EPL diagnosed

clinically and radiographically and with indication of extraction were extracted under local anaesthesia. In addition to severe EPL, the teeth had either deep circular periodontal defects (pocket depth over 7 mm) with increased mobility, or advanced furcation involvement, or extensive carious destruction. All teeth were asymptomatic and had no fistula. For each tooth, the following data were recorded: the type of EPL (based on the case history), the position of the periodontal pocket, the presence/absence of vitality, the existence of a root canal filling, the pocket depth, the clinical attachment level, the gingival recession, the plaque index PlI (Silness & Loe, 1964), the bleeding on probing BOP, the furcation involvement (Glickman, 1950), and the mobility (on the Miller scale).

After extraction, the samples were carefully and gently rinsed with sterile saline solution, in order to avoid the disruption of the biofilm and to remove any biological material that could possibly come in contact with the root during the extraction (e.g., blood). The samples were prepared according to the protocol described by Noiri and Ebisu (2000), modified for examination in low-vacuum SEM. For all further manipulation of the samples, delicate pliers were used only on the coronal third of the roots, in order not to disrupt the biofilm. The samples were introduced in vials for fixation in modified Karnovsky solution (glutaraldehyde 2.5%, paraformaldehyde 4%, sodium cacodylate 0.1 M at pH 7.2-7.4); the transportation of the vials to the laboratory took utmost care to prevent as much as possible the samples to touch the walls of the vials. The samples were dehydrated in series of ethanol (70, 95 and 100%), changed every 15 min. Because the prolonged immersion in 100% ethanol could irreversibly modify the aspect of the biofilm through extreme dehydration, only 3 samples at a time were dehydrated and then immediately examined under low-vacuum SEM in the laboratory using the SEM Inspect S (FEI), under pressures of 80-250 Pa and acceleration voltage of 15 kV. For samples with higher conductivity, the pressure used was 80 Pa, while for those with lower conductivity the pressure used was 150 Pa, as the conductivity is known to increase with the density of the examined biologic material.

Before SEM examination, all samples underwent a preliminary examination under a light microscope. The primary examination was performed under magnification x50-x80, in order to localize the apical foramen or to select the main apical foramen in case there was more than one. The magnifications x200, x500 and x800 were used for the examination of the external radicular surface, for the areas of cemental and dentinal resorptions, and for the detection of the presence of the bacterial film. Finally, the magnifications x1,000-x20,000 were used for the detection and characterization of the morphology of the microorganisms. The bacteria included in the biofilm, as well as the solitary microorganisms on the hard surfaces were morphologically categorized in cocci, rods, motile (spirochete, spirils) and filaments. Through graphic delimitation of specific areas of pictures and using the Print screen function, the objects of interest were identified. The chronological list of these areas under increasing magnification was saved on a single Word document and registered under the tag of each sample, to make sure the identification of each object of interest can be re-traced at any time later.

For the SEM topographical examination of the biofilm, target zones on the apical surface of each sample were defined as follows: The internal wall of the cemental cone (its biofilm mostly seen as an extension of the root canal infection, sometimes in the presence of root canal filling materials); the near-foraminal (peri-foraminal, juxta-foraminal) zone (present in any typical chronic apical infection); the ‘transition’ zone between the near-foraminal and the periodontal pocket zone (of great interest in the hypothesis that it harbours biofilm with mixed morphology: Endodontic and periodontal); the periodontal pocket zone (harbouring typical periodontal biofilm).

The ‘transition’ zone was considered to be limited apically by the marks of the former apical lesion (cemental resorptive lacunae for the cases of EPL with primary endodontic

onset and for the very rare cases of simultaneous EPL) and coronally by the apical limit of the calculus deposits typical for periodontal pockets. For EPL with primary periodontal onset and no marks of resorption available, the 'transition zone' was considered to begin at 2 mm coronally to the crest of the cemental cone.

As the understanding of the radicular biofilm morphology needs a preliminary 'inspection' phase, a collection of characteristic SEM images was created, in order to provide typical visually recognizable elements for further reading of the images of the samples. Figures I.1-I.20 represent a selection of the most relevant images for the present study, included in the collection.

On all 5 zones, the following elements were qualitatively evaluated: The established biofilm, the glycocalyx matrix, the presence of isolated microorganisms, the relative presence of microbial morphologies - cocci, rods, filamentous forms, motile forms (spirochetes, spirilli), areas of nude cementum, lacunae of cemental resorption, the presence of calculus, the presence of unstructured (amorphous) material (debris), the presence of the root canal material (in cases with endodontic treatment), the presence of red blood cells as result of the extraction procedures (as they can mask the biofilm). The presence of these elements varied, depending on the zone. Specific elements noted in the near-foraminal zone were the periodontal ligament fibres, in various degrees of decomposition, depending on the vicinity with the EPL. In the near-foraminal zone, the transition zone and the periodontal pocket zone, the biofilm was sometimes found populating the interior of the cemental lacuna.

Depending on their quantity on the studied SEM images, the data were recorded by the examiner to the following categories: (score 0), absence; + (score 1), + 'low quantity'; ++ (score 2), 'significant quantity'; +++ (score 3), 'abundant'. The scores were used to establish statistical correlations.

The resorptions (cemental lacunae) were separately analysed, as they are considered zones of special agglomeration of the biofilm, by offering a particular shelter to the microorganisms. The analysed elements in the resorption areas were: The character of the resorptions (isolated, multiple, generalized); their near-foraminal presence (as indicating an old apical lesion), the SEM appearance of the lacunar relief (apparently shallow, apparently deep), the presence of the established biofilm, the predominant morphology of the bacteria (cocci, rods, filaments, motile species), the presence of isolated bacteria. These values were qualitatively and quantitatively evaluated, as well, as described before.

The qualitative data for biofilm characteristics on all EPL zones was summarized by computing rates of prevalence. The relations between the biofilm characteristics assessed semiquantitatively were investigated using non-parametric correlational analysis (Spearman rho correlation coefficients and corresponding significance tests performed using significance level α=0.05). The data were analysed using the software R version 4.0.0.

Results

Four out of 25 samples were eliminated during the primary microscopic examination due to following reasons: Apex fully covered with calculus and no detectable apex, the complete absence of the biofilm and microorganisms (due possibly to incorrect manipulation of the sample), the abundant presence of residual periodontal fibres that prevented the determination of the target zones on the root surface. Thus, 21 teeth entered the examination. A total number of 44 images were selected for their quality and special relevance and were included in a separate collection (Figures I.2-I.21). In all samples, the cementum presented near the apical foramen apparently shallow or deep areas of resorption of various shapes and dimensions, some including clusters of agglomerated, inserted residual collagen fibres. Within the biofilm in these cemental lacunae, microorganisms were present, either monomorphic (cocci, rods, filaments, motile forms), or in association. In some specimens,

small resorption areas, containing biofilm, were noted on the intact cementum. Only 5 (24%) teeth with EPL included in the study presented a mature biofilm on the inner surface of the cemental cone, and 2 (9.5%) presented isolated microorganisms, 38% cocci and 5% rods. In 3 samples (14%), the matrix (glycocalyx) with few microorganisms was observed on the inner wall of the cemental cone.

On the near-foraminal zone, mature biofilm was found in 4 out of 21 samples (19%) and isolated microorganisms only in 1 sample (4.7%). The identified microorganisms were in 28.5% of the samples cocci, in 9.5% of the samples rods, motile forms (spirilli) in only 1 sample (4.7%). In 1 sample a matrix poor in microorganisms was found, and in another 1 sample isolated microorganisms.

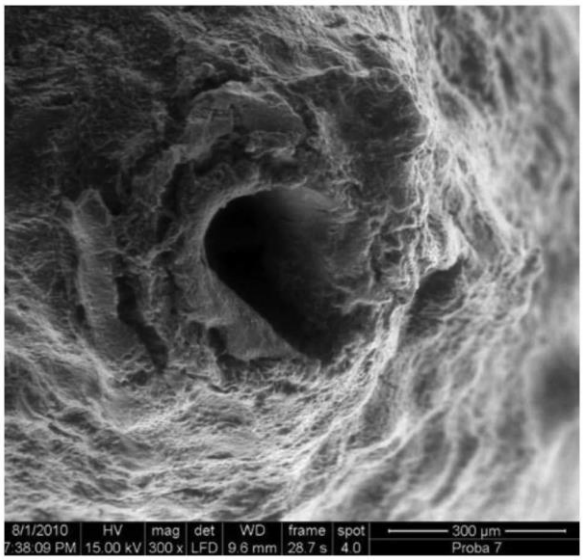


Figure I.2. Internal wall of the cemental cone, x300.

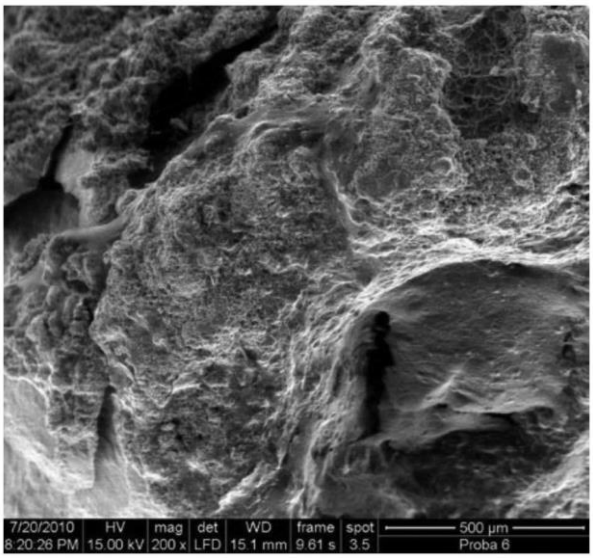


Figure I.3. Near-foraminal zone, x200

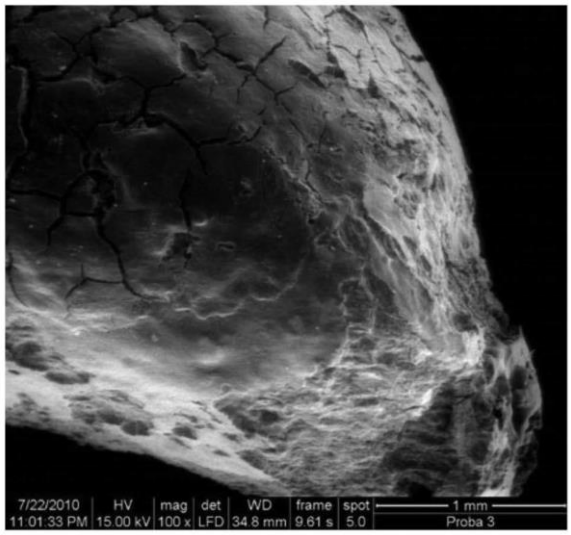


Figure I.4. The 'transition' zone x100.

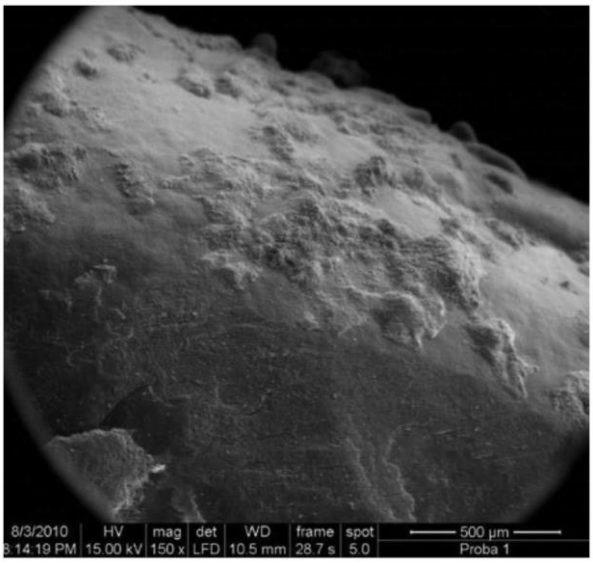


Figure I.5. The radicular surface inside the periodontal pocket. Note the typical calculus covered with biofilm, x150.

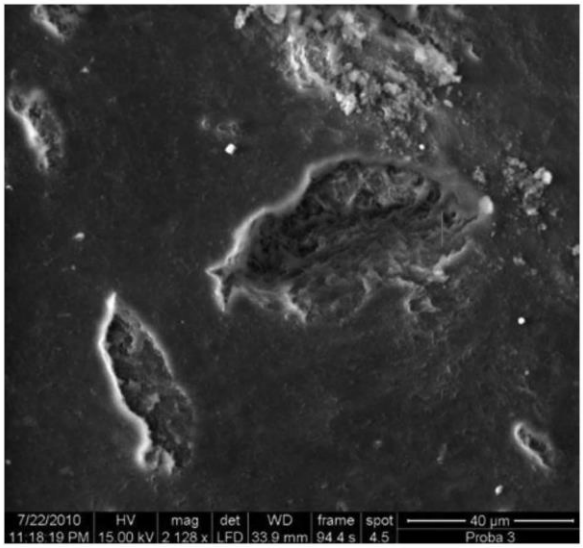


Figure I.6. Isolated cemental lacunae on the root surface in the former periodontal pocket, x2,120

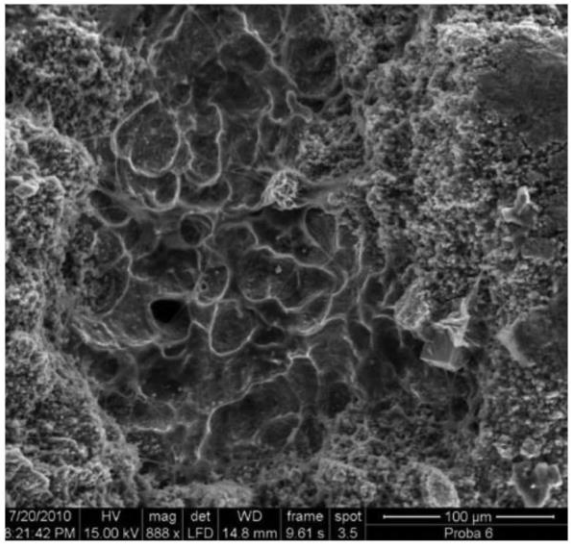


Figure I.7. Multilocular cemental resorption (lacuna) surrounded by abundant biofilm. Filaments of glycocalyx can be noted across the lacuna, x890.

In the 'transition' zone, mature biofilm was found in 8 out of 21 samples (38%) and isolated microorganisms in 5 other samples (24%). In 57% of the samples cocci were identified and in further 24% rods, filaments in 24% and no motile forms were found. In this zone 6 out of 21 samples (28.5%) presented marked resorptions; in 3 samples (14%) the resorption lacunae were populated by biofilm. In 24% of the samples, the 'transition zone' presented residual periodontal fibres and 24% presented calculus.

In the periodontal pocket zone, especially on the calculus deposits, the biofilm percentage increased to 52.3% of the samples, 62% of these showed cocci, 38% rods, 23.8% filaments but, surprisingly, no sample presented motile forms. Less resorptions were found (4.7%), residual periodontal fibres (38%) and isolated bacteria (14.2%).

The special separated analysis, of the biofilm in the cemental resorption lacunae, using the same criteria, revealed in 24% of the samples thick, mature biofilm (5 out of 24 samples), 52% presented cocci, 19% rods and no motile form or filament. Figure I.21 shows the frequency of the distributions of all investigated parameters, in all zones of interest.

The statistical analysis of the data revealed the presence of mature biofilm on the inner wall of the cemental cone in 24% of the samples; this drops to 19% in the near-foraminal zone and then increases to 38% in the 'transition zone' and to 52.3% in the periodontal pocket zone. Abundant quantity of biofilm was found only in the 'transition zone' (9.5%) and in the periodontal pocket zone (19%).

The extracellular matrix (glycocalyx) poor in bacteria was found on the inner wall of the cemental cone in 14% of the samples, on the near-foraminal zone in 4.7% of the samples, in 9.5% in the 'transition zone' and more frequently in the periodontal pocket zone (14.2%). An appreciable amount of extracellular matrix poor in microorganisms was found only in the 'transition zone' in 9.5% of the samples.

Isolated microorganisms were found in 9.5% of the samples on the inner wall of the cemental cone, 4.7% in the near-foraminal zone, in 24% of the samples in the 'transition zone' and in 14.2% of the samples in the periodontal pocket zone. In the 'transition zone', in 9.5% of the samples, the quantity of isolated microorganisms found was abundant.

The coccoid morphology was found in 38% of the samples on the inner wall of the cemental cone, in 28.5% in the juxtaforaminal zone, in 57% of the samples in the 'transition zone', and in 62% in the periodontal pocket samples. Abundant accumulations of cocci were found in the near-foraminal zone in 4.8% of the samples, in 19% of the samples in the

‘transition zone’ and in 14.3% of the samples in the periodontal pocket zone.

The proportion of the rods increased from 5% of the samples on the the inner wall of the cemental cone to 9.5% in the near-foraminal zone, 14% in the ‘transition zone’ and to 38% in the periodontal pocket zone. An abundant quantity was found only in the periodontal pocket zone, in 4.8% of the samples. Filamental morphology of the bacteria was found neither on the inner wall of the cemental cone zone, nor in the near-foraminal zone, but in 24% of the samples on the ‘transition zone’ (4.8% in abundant quantity) and in 23.8% of the samples in the periodontal pocket zone.

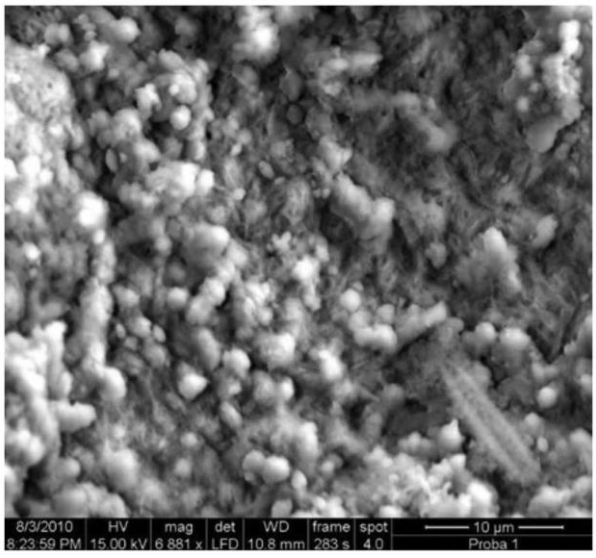


Figure I.8. Detail of established biofilm, including glycocalyx, coccoid microorganisms and a large rod-like bacterium, x6,900.

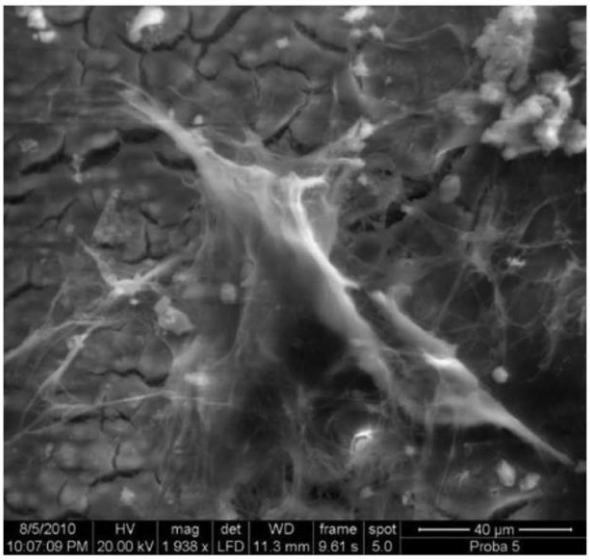


Figure I.9. Isolated fragment of glycocalyx on the cemental surface, poor of bacteria, a probable result of early colonizers, x1,900.

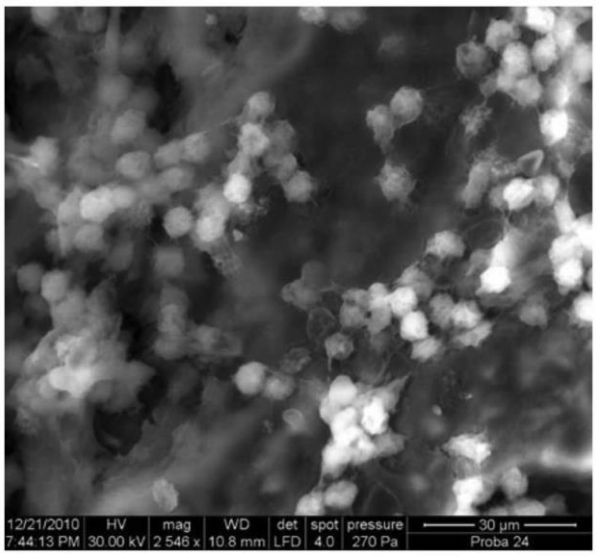


Figure I.10. Low-density coccoid bacteria (early colonizers?), included in a minimal glycocalyx. Note the glycocalyx attachment strings to the cemental surface, x2,500.

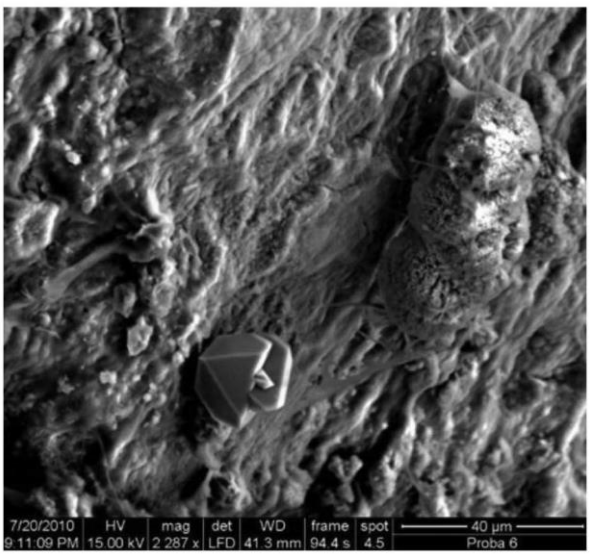


Figure I.11. Torsade-shaped rod colony and a polyedric inorganic crystal on the root surface in the periodontal pocket, x2,300.

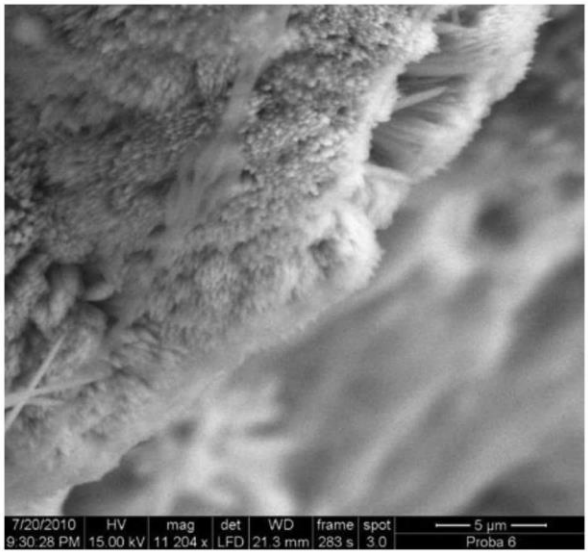


Figure I.12. Detail of the torsade-shape colony, x11,200.

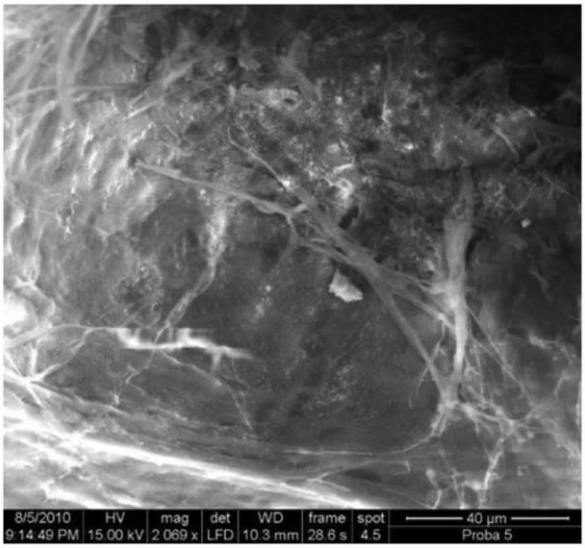


Figure I.13. Isolated residual periodontal fibres near the apical foramen, x2,100.

Under SEM observation, motile forms (spirils/spirochetes) were found only in the near-foraminal zone in 4.7% of the samples in reduced quantity (4.8% of the samples). The cemental resorptions were found in 14% of the samples on the inner wall of the cemental cone, in 43% of the samples in the near-foraminal zone, in 28.5% in the 'transition zone' and 4.7% in the zone of the periodontal pocket. Biofilm was found inside the resorption lacunae in 4.7% of the samples in the near-foraminal zone, 14% in the 'transition zone' and 23.8% in the periodontal pocket zone.

Calculus was not found on the inner wall of the cemental cone, but it was found in 4.7% of the samples in the near-foraminal zone, in 24% of the samples in the 'transition zone', and in 81% of the periodontal pocket zone samples. In 9.5% of these samples, the quantity found was considered abundant. The low-vacuum SEM examination of bacterial biofilm on root surfaces proved useful in the present study because the method allowed the preservation of samples and avoided high electrostatic loads during examination. The sample preparation method for low-vacuum SEM examination differs from the usual SEM preparation method for biologic samples, in which the dehydration of the samples is completed by using CO_2 in drying devices at a critical point, followed by fixation of the samples with adhesive conductive silver on metallic support discs, and the sputtering of samples with 5-10 nm of pure gold to make them conductive. This protocol is described minutiously by Leonardo et al (2002), indicating a thickness of 200 µm of the gold sputtering coating.

Another protocol completes the dehydration of the samples in a lyophilisation device using t-butylic alcohol, and sputters the samples with osmium oxide with a 5A thick conductive layer obtained with a plasma-multicoater device (Noiri et al., 2002). Both described methods are technique-sensitive and expensive.

In this study, the dehydration procedure in increasing alcohol concentrations was completed with a light air-blow for a few seconds. The dehydration was maximized by lowering the pressure inside the microscope vat up to 80-250 Pa (low-vacuum). This procedure extracts all alcoholic remnants from the samples, carefully and at slow pace. Despite the careful induction of the low-vacuum, several samples presented signs of biofilm disruption, bacterial body damage and root surface cracks, indicating further need for a better control of the experimental procedure.

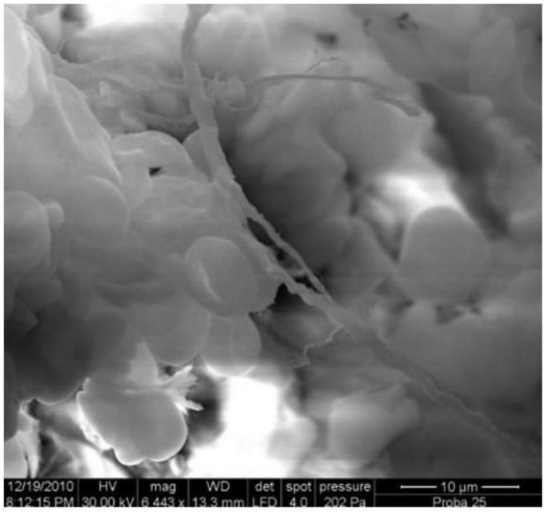


Figure I.14. Isolated spyrils and red blood cells, x6,400.

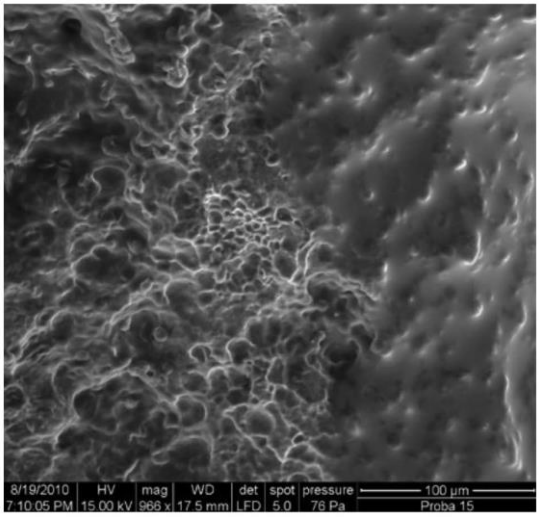


Figure I.15. Normal cement adjacent to an area of resorption, x1,000.

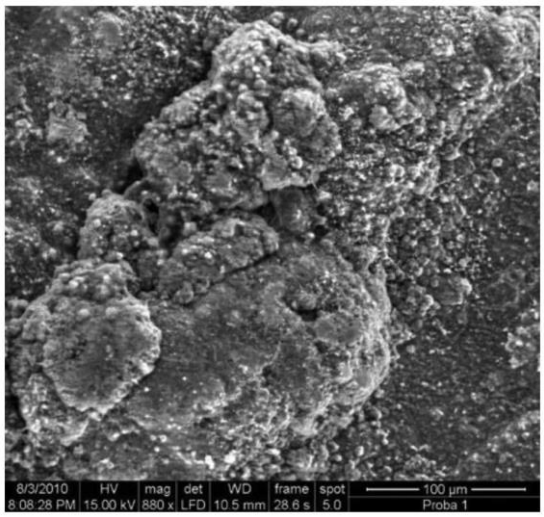


Figure I.16. Massive thick calculus deposit in the periodontal pocket, x900.

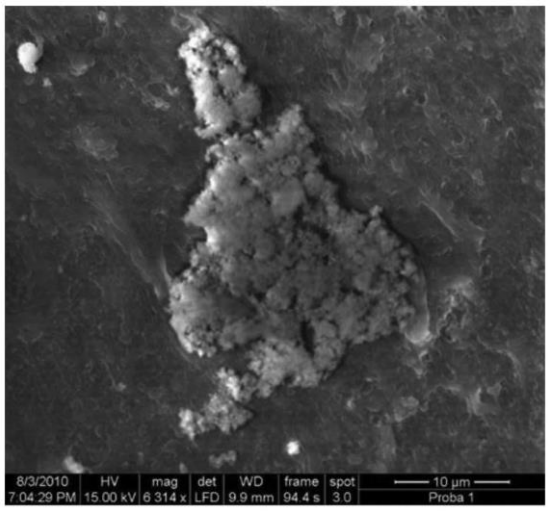


Figure I.17. Unstructured amorphous debris on the nude cemental surface, x6,300.

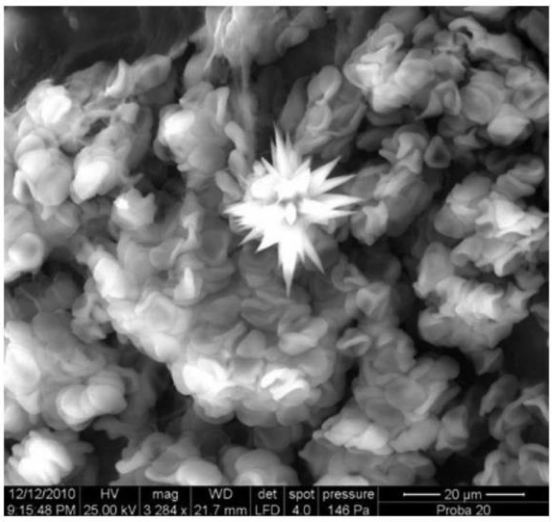


Figure I.18. Star-like crystal (talcum crystal?) fixed over a dehydrated blood clot, x3,300.

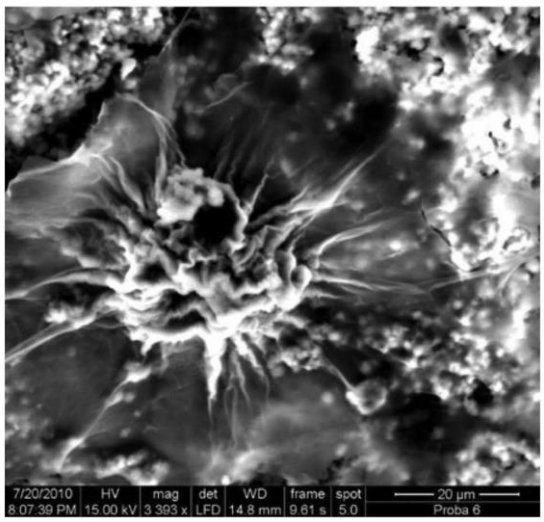


Figure I.19. Efflorescent circular patch of glycocalyx, including later microcolonies, layered over previous abundant biofilm, x3,900.

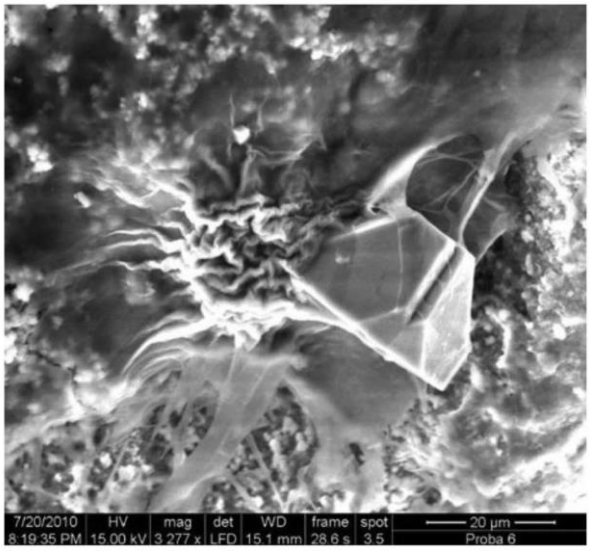


Figure I.20. Recent efflorescent superficial microcolony, including a polyedric crystal, x3,300.

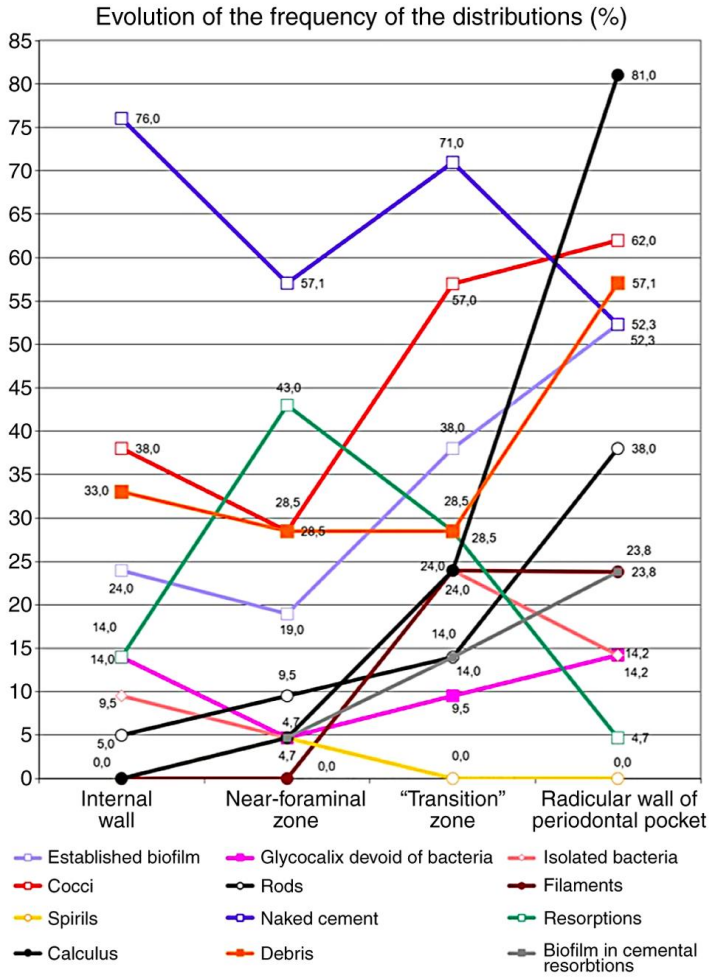


Figure I.21. Synthetic graphic representation, with linear markers of the frequency of the distributions of all investigated parameters, in all zones of interest.

Discussion

The SEM observations focused on a root surface including the continuum root surface inside the periodontal pocket - a so-called 'transition zone' (between the apical part of the root surface inside the periodontal pocket and the former initial apical lesion, corresponding to the former confluence zone between them), the near-foraminal zone (the surface of the apex inside the former apical lesion), the inner wall of the cemental cone. Part of the continuum described above is the 'plaque-free-zone' (PFZ) described by Brady (1973), a near-apical root zone situated in the periodontal pocket underneath the most apical extension of the calculus tartar, where bacteria organized as biofilm (plaque) tend to disappear, as a consequence of the permanent and sustained cell defence of the host near the epithelial junction. In our experiment, it was considered that the conventional zones delimitated as above are potentially containing biofilm with noteworthy individual characteristics.

The delimitation of the observation zones on the root surface was conventional and not without difficulties. The morphology serving to delimitation of both the inner wall of the cemental cone and the near-foraminal zone was relatively clear. The 'transition' zone was apically delimited by the typical landmark of the former apical lesion (cemental resorptions with typical texture and clear limits in EPL with primary endodontic origin and in the rare case of combined simultaneous EPL) and coronally by the apical limit of calculus deposits existent in the periodontal pockets. In the case of primary periodontal EPL, the 'transition' zone was conventionally defined as starting at 2 mm distance from the periforaminal crest. In this study, the endo-periodontal microbial population organized as a biofilm, was studied in all 4 conventional zones described: The inner wall of the cemental cone, the near-foraminal zone, the 'transition' zone, the periodontal pocket zone. The observations followed the variations of distribution of characteristic morphological elements of the biofilm in a continuous mode, from the terminal zone of the root canal to the radicular wall of the periodontal pocket. A first conventional division of the peri-foraminal region of apices with pulpal infections and apical lesions of pulpal origin is known in the literature as 'extraradicular zone'. This described the apical external zone, outside the root canal (Noguchi et al., 2005). Although the 'extraradicular zone' is situated inside the chronic periapical lesion, it is considered distinct of the lesion. The lesion represents the volume of resorbed alveolar bone around the apex, which often contains granulation tissue. The approximately 2 mm area around the apical foramen (designated in our study as 'near-foraminal' or 'juxta-foraminal' zone) was separately investigated only in one study (Molven et al., 1991).

The morphology of the bacteria and biofilms on apices associated with refractory and chronic periapical periodontitis was thoroughly investigated in a classic study (Noiri et al., 2002). Another SEM study of the periodontal biofilm investigated seven perio-pathogenic bacteria in the biofilm of the 'plaque-free zone' (PFZ) by scanning immunoelectron microscopic techniques, using both secondary and back-scattered imaging, with rabbit antibodies specific for each bacterium (Noiri & Ebisu, 2000). Starting from the concept of PFZ, in our study the limits of the 'transition' zone were defined between the bottom of the former periodontal pocket and the former apical lesion.

In our study, the frequency of detection of mature biofilm was found only in 24% of the samples on the inner wall of the cemental cone, in 19% of samples in the near-foraminal zone (zone corresponding to the former endodontic lesion), and is constantly increasing with distance to the apical foramen: 38% in the 'transition' zone and 52.3% in the periodontal pocket. In the literature, available data vary greatly. A study on 21 extracted teeth found apical biofilm and microorganisms in all investigated samples with pulpal necrosis and radiographically visible lesions (Leonardo et al., 2002). Mature extraradicular biofilm was found in 20 out of 27 patients with refractory apical periodontitis (Noguchi et al., 2005). Finally, established biofilm was found on 106 roots with apical periodontitis bacteria, with

only one exception. The same study noted that the presence of biofilm in cysts, abscesses and granulomas was 95, 83 and 69.5%, respectively.

In addition to the data found in the current literature, (e.g., the frequency of presence of mature biofilm), our study recorded various other relevant data: the matrix poor in microorganisms, the presence of isolated microorganisms, semi-quantitative data on the composition of the biofilm with respect to the microbial morphology (cocci, rods, filaments, motile forms), the presence of resorptions, of calculus, of periodontal residual fibres, of amorphous debris.

The existing literature does not present data regarding the presence of the different bacterial morphologies in the apical and periodontal biofilm. A classic study observed a morphological variety of cocci, rods and filaments, as well as associations between rods and filaments (Leonardo et al., 2002). These results matched the results of another study, that found bacteria, yeasts and biofilm in the vicinity of the foramen, in the areas of radicular resorption and on the external surface of human teeth apices with pulpal necrosis and chronic apical lesions (Lomçali et al., 1996). Some later studies identified the bacterial species in the biofilm with PCR immunohistochemical methods, insisting on the role of *P. gingivalis* in the primary extraradicular colonization (Noguchi et al., 2005). Our SEM study shows semi-quantitative data on the relative proportion of the coccoid morphologies (cocci being considered primary colonizers) in the 4 experimental zones. Abundant accumulations of cocci were found in the near-foraminal zone in 4.8% of the samples, in 19% in the 'transition' zone and, in 14.3% of the samples in the periodontal pocket zone, and very little on the inner wall of the cemental cone.

Rods were found in our study in 5% of the samples on the inner wall of the cemental cone, 9.5% in the near-foraminal zone, 14% in the 'transition' zone and to 38% in the periodontal pocket zone. An abundance of rods was found only in the periodontal pocket zone in 4.8% of the samples in agreement with literature data (Noiri & Ebisu, 2000). Coccoid microorganisms were observed in this study mostly on the biofilm surface (probably during release phases from clusters), and in monomorphic and mixed agglomerations. In an *in vitro* study on gutta-percha cones, it was found that cocci were localized in deeper biofilm layers, as they play an important role in the biofilm initiation (Takemura et al., 2004).

As a general observation, in our study we found a common variation of detection frequency of all investigated characteristics: a slight decrease or no change from the inner wall of the cemental cone to the near-foraminal zone, followed by a slight raise towards the transition zone, and a more pronounced increase towards the periodontal pocket zone.

Of special interest for our research was the distribution of cemental resorptions and their population with isolated and aggregated bacteria. This study includes observation of SEM characteristics of cemental resorptions in EPL, important for biofilm formation and persistence (e.g., frequency, relative depth, localization, fibre- and biofilm content). The incidence of radicular resorptions as potential sites for persistent biofilm on 39 apical thirds of extracted teeth was evaluated in a recent study (Felippe et al., 2009).

The images in this study show the existence and persistence of bacterial colonies organized as biofilm also in fissures of hard radicular surfaces, in cementum and calculus deposits. The decisive influence of the surface roughness and free superficial energy on the bacterial adhesion and biofilm formation is demonstrated in this study by the relation between the frequency of calculus distribution (in 4.7% of the samples in the juxta-foraminal zone, in 24% in the 'transition' zone samples, and in 81% of the periodontal pocket zone samples, as expected) and the presence of mature biofilm: the comparison of the graphics of the two distributions reveals the increase from the apical foramen to the periodontal pocket. Despite this, in our study we found areas covered by calculus but free of biofilm, even inside the periodontal pocket, demonstrating possible biofilm disruptions during the preparation of

the samples.

The presence of residual periodontal ligament fibres and their relation with the biofilm microorganisms in the apical region was recently studied in a group of 18 teeth (Rocha et al., 2008). In teeth with normal healthy pulp and with necrotic pulp but without radiographic visible apical lesions, the apical surfaces were covered with collagen fibres in the total absence of bacteria, whereas in necrotic teeth with radiographic visible lesions, the apices did not present collagen fibres, but areas of resorption with microorganisms were found in all samples. In contrast with these findings, the present study on EPL found residual periodontal ligament fibres in 14.2% of the samples in the juxta-foraminal zone, in 24% of the samples in the 'transition' zone and in 38% of the samples from the periodontal pocket zone.

Rods and filaments were correlated to the presence of rough calculus surfaces. Generally speaking, the strong correlation between mature biofilm and the presence of cocci appears in all investigated zones, while the presence of rods and filaments appeared to depend on the roughness of the surfaces (calculus and cemental resorptions).

Data in our study indicate a strong correlation between the microbial flora of the three EPL zones, regardless of the organization form of the microflora (biofilms or isolated bacteria), provided rough surfaces due to calculus or resorptions are available.

The SEM investigation of the radicular surfaces involved in EPL in our study revealed less surfaces covered by biofilm than expected. There is no explanation for this observation, knowing also that the resistance to antimicrobial therapy of EPL is attributed to the persistence and inaccessibility of the biofilm.

Limitations of the present study include the conventional choice of the radicular zones and of the morphological characteristics of the biofilm. The interpretation of the results was done with caution, because of the age of the EPL, the frequency of acute episodes in apical lesions and periodontal pockets and the risk of biofilm destruction during sample preparation.

Conclusions

The SEM investigation of radicular surfaces involved in EPL revealed less surfaces covered by biofilm than expected. The microbial morphologies described by the present SEM investigation were mostly coccoid forms, seldom rods or filaments. Spirochetes were found only accidentally. These findings are contradictory to literature data. Our data showed that the mature biofilm appears to be associated with the roughness of the support, due especially to the presence of cemental resorptions and calculus. Despite the communication between the periapical lesion and periodontal pocket, the biofilm elements seem to be better represented in the periodontal pocket than in other zones of the EPL.

I.5 The importance of periodontal evaluation in cases of orthodontic treatment

State of the art in the orthodontics-periodontics relationship

In patients with a history of periodontitis resulting in displaced teeth, possible orthodontic tooth movements include changes in alignment, space redistribution, and intrusion. The primary aim, before orthodontic intervention might start, is to stabilize the periodontal condition. Bone loss alters the position of the tooth's centre of rotation and the force required to achieve the movement; however, the orthodontist can use reduced or increased force moments to avoid excessive alveolar bone loss.

Orthodontic therapy has been shown to be a reliable therapy for restoring compromised dentition, closing infrabony defects, reducing gingival recessions, and improving interdental papilla levels; thus, orthodontics can be considered for the treatment of periodontal patients with tooth migration (Cardaropoli, 2009). Other studies have shown that orthodontic treatment can safely be used in patients with previous periodontal therapy, despite the fact

that orthodontic appliances worsen conditions for oral hygiene, complicate tooth care, and, thereby, create an environment favourable to plaque accumulation (Zharmagambetova et al., 2017). However, orthodontic treatment often employs the permanent or long-term use of a retainer which can complicate dental hygiene self-cleaning procedures, and can potentially harm the periodontal tissues (Zharmagambetova et al., 2017). In time, periodontal parameters can deteriorate, even if some of them may improve after the removal of the orthodontic retainers (Van Gastel et al., 2011). A systematic review showed a deterioration of periodontal parameters after orthodontic treatment, indicating that it influences the accumulation and composition of the subgingival microbiota and subsequently induces more inflammation and higher BOP (Verrusio et al., 2018).

Earlier studies hypothesized that the orthodontic treatment can improve or prevent deterioration of periodontal parameters in treated periodontal patients. Significant reductions in pocket depth (PD) and clinical attachment levels (CAL), as well as radiographical improvements of periodontal bone defects were reported (Corrente et al., 2003). Since orthodontic therapy can be safely used in patients with previous periodontal therapy, it is only coherent to use it as an additional tool in periodontitis treatment, even if the inherent risks of the therapy must be taken into consideration (Bollen et al., 2008).

Therapeutic setbacks as ‘black triangles’ and reduced interdental papilla heights (Gorbunkova et al., 2016) and difficult prosthetic rehabilitation can be prevented in orthodontic patients with previous periodontal disease, together with the additional attachment loss, through strict biofilm control and periodontal maintenance. These procedures are essential during the active phase of orthodontic treatment, in order to prevent inflammation in gingival tissues (Carvalho et al., 2018).

Bone changes induced by orthodontic treatment may impact the morphology of bone defects, decrease pocket depth, and enhance connective tissue healing (Gorbunkova et al., 2016). The influence of tilting movements in the presence of infrabony pockets is further evidence that orthodontic movements may be performed in teeth with bone defects without further damaging periodontal attachment (Cirelli et al., 2003). Thus, orthodontic forces must be carefully applied in teeth with a reduced periodontium.

Despite the high number of published articles debating the periodontal-orthodontic interrelationship, there is a lack of good evidence on systematic treatments including both orthodontic and periodontal therapy. The periodontic-orthodontic interrelationship has been the subject of substantial investigation, yet it remains a controversial issue (Meeran & Parveen, 2011). Thus far, the literature regarding orthodontic treatment in subjects with treated periodontal disease is represented mostly by case reports on subjects already treated for chronic periodontitis (Carvalho et al., 2018). Of more interest for clinical studies, however, might be the relationship between aggressive periodontitis and orthodontic treatment, probably because of the significant tooth displacement related to this rapidly progressing form of periodontitis (Khorsand et al., 2013). In the early 2000's, the Cardaropoli group (Re et al., 2004) reported that orthodontic treatment is no longer a contraindication in the therapy of severe adult periodontitis. While orthodontics has improved the ability to restore deteriorated dentition, over the last decade there has been no clinical study regarding the outcome of treated periodontium undergoing orthodontic movements. There are also relatively few clinical studies comparing the outcomes of combined periodontal-orthodontic treatment with the outcomes of periodontal treatment alone in patients with severe periodontitis (Boyer et al., 2011).

This research direction has been materialized by publishing the following papers:

1. Calniceanu H, Stratul SI, Rusu D, Jianu A, Boariu M, Nica L, Ogodescu A, Sima L, Bolintineanu S, Anghel A, Milicescu S, Didilescu A, Roman A, Surlin P, Solomon S, Tudor M, Rauten AM. Changes in clinical and microbiological parameters of the periodontium during initial stages of orthodontic movement in patients with treated severe periodontitis: A longitudinal site-level analysis. Exp Ther Med. 2020 Dec; 20(6): 199.
https://pubmed.ncbi.nlm.nih.gov/33123229/
2. Sioustis IA, Martu MA, Aminov L, Pavel M, Cianga P, Nitescu-Kappenberg DC, Luchian I, Solomon SM, Martu S. Sallivary metalloproteinase-8 and metalloproteinase-9 evaluation in patients undergoing fixed orthodontic treatment before and after periodontal therapy. Int. J. Environ. Res. Public Health 2021; 18(4):1583.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7915089/

I.5.1 Changes in clinical and microbiological parameters of the periodontium during initial stages of orthodontic movement in patients with treated severe periodontitis

Aim of the study

We conducted a research with the aim to determine the longitudinal changes in clinical and microbial parameters of the periodontium at site-level during the initial remodelling processes of the alveolar bone and periodontal ligament caused by light continuous forces employed in the orthodontic treatment of adult patients with a history of severe periodontal disease treated with standard (non-surgical and conventional non-regenerative) periodontal therapy.

Materials and method

Thirteen adult patients (8 women and 5 men, aged 23-53 years, mean age 36.5 years), with a history of severe periodontitis as described by Armitage (1999) treated with standard (initial and conventional surgical) periodontal therapy received fixed orthodontic appliances. The criteria for inclusion in the study were: i) ≥21 y/o; ii) good systemic health in terms of diabetes, cardiovascular diseases and other conditions that may impact the periodontal status; iii) absence of extended fixed and removable prosthetic restorations; iv) no previous orthodontic treatments; v) severe periodontitis treated by standard (non-surgical and conventional surgical) procedures, the treatment being completed at least one year before the onset of the orthodontic treatment; vi) good compliance with a rigorous (with respect to the initial 3-months recall intervals, good personal oral hygiene), supportive periodontal therapy; vii) stable periodontal status during the previous six months (absence of inflammation and attachment loss); viii) good oral hygiene (full mouth plaque and full mouth bleeding scores under 25%); ix) teeth affected by periodontal attachment loss, misaligned or displaced following the evolution of periodontitis and x) indication for orthodontic treatment for aesthetic or functional reasons. Exclusion criteria were administration of antibiotics during in the previous six months, pregnancy, lactation, smoking, allergies to the materials included in the orthodontic appliances, incapacity to read and understand the aim and nature of the study. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

Clinical measurements and subgingival plaque sampling were performed for each individual patient on the same tooth and site by the same intra-examiner calibrated investigator (AJ). The selection criteria for experimental periodontal sites were: i) residual PD ≥3 mm; ii) situated on single-rooted teeth; iii) at the site where the periodontal ligament underwent compression. Experimental teeth underwent orthodontic corporeal movements predominantly in mesial direction (12 teeth) and distal direction (one tooth). In each

experimental tooth, at the periodontal site that displayed the deepest residual pocket at the beginning of the orthodontic treatment, the periodontal clinical status was evaluated at baseline and at 2, 4, and 6 month intervals; microbiological status was evaluated at baseline and again after 6 months. Gingival crevicular fluid (GCF) sampling for determining enzymatic and inflammatory changes during the orthodontic treatment was performed on the same experimental sites (data to be published elsewhere). Orthodontic treatment was initiated 12 months after the completion of the planned active periodontal therapy, even if a small number of pockets deeper than 3 mm persisted, within a well-controlled periodontal maintenance program.

Twelve months after the end of periodontal treatment, orthodontic brackets (Omniarch®, Dentsply GAC), slot 0.018 inch and Sentalloy Superelastic® (Dentsply GAC), size 0.014 inch wires were applied to each patient with a Roth Rx prescription.

In each selected site of the experimental teeth, the following parameters were evaluated: PD (pocket depth), REC (gingival recession), CAL (clinical attachment level), BOP (bleeding on probing), and PPI (papilla presence index). The PlI (plaque index) was evaluated in each patient in the experimental tooth only. The measurements were made before applying the orthodontic braces (T0) and again at 2, 4, and 6 months after orthodontic treatment with the exception of PPI, which was assessed at baseline and after 2 and 4 months of orthodontic treatment.

The presence of five main periodontopathic bacteria in the gingival sulcus was evaluated at baseline and after 6 months: *Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Tannerella forsythia (Tf),* and *Treponema denticola (Td).*

Sampling was performed after complete removal of the supragingival plaque and isolation of the tooth with cotton rolls. The tooth was dried with gentle air flow in order to avoid contamination of the samples with saliva. At each site, two #30 0.04 sterile paper points (Roeko GmbH) were inserted and held in place for 30 sec. until soaked. After sampling, the cones were transferred to Eppendorf tubes containing 700 μl PBS solution and kept refrigerated in a special thermo-isolated box during the transport to the laboratory. Each tube containing plaque sample received a code. It was vortexed for 30 sec. at room temperature. The points were removed and the elutes clarified by centrifugation for 5 min at 3,000 x g at 4˚C. Samples were stored for one day at -20˚C and then at -80˚C until microbiological analysis (no longer than a month). The processing of the samples included DNA extraction, amplification, and hybridization. For DNA extraction the QIAamp DNA Micro kit (Qiagen GmbH) was used, in accordance with the manufacturer's instructions. Sample DNA hybridization was performed with a micro-IDent plus® kit (Hain Lifescience GmbH). For amplification, a HotStar Taq Polymerase kit (Qiagen GmbH) was employed; this is an inactive polymerase that offers high specificity for PCR and facilitates the amplification process by eliminating several reaction steps. Amplification was performed using a thermo cycler (Thermo Fisher Scientific Inc.) for 32 cycles. After hybridization, the reading strips were submerged in the sample tube and were incubated at 45˚C for 30 min. Extracted DNA was quantified by spectrophotometry (230 nm), using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). Accordingly, semi-quantitative data were recorded.

Results

The changes in mean PD, REC, and CAL between the measurements at different time points are given in Table I.4 There were no statistically significant differences between the measurements at different time points. For PPI, there were no significant differences between successive time points (Friedman test, P=0.36). At each time point, PPI values ranged from 1

to 3 (median=2). For 12 out of 13 patients, no PPI changes occurred between the three observation time points. For one patient, PPI increased from 2 to 3 in the first interval, but remained unchanged afterwards (Fig. I.22).

Table I.4. Descriptive statistics for PD, REC, CAL, and PlI measured at baseline and at 2, 4, and 6 months of orthodontic treatment (mean ± SD, range specified in parentheses)*

Parameter	Baseline	2 months	4 months	6 months	P-value
PD	4.23±1.09 (3-7)	3.77±1.24 (2-7)	3.92±0.86 (2-5)	4.00±0.82 (2-5)	0.412
REC	0.77±1.01 (0-3)	0.77±1.01 (0-3)	0.85±0.99 (0-3)	0.69±0.95 (0-3)	0.494
CAL	4.92±1.50 (3-8)	4.54±1.66 (2-8)	4.77±1.42 (3-8)	4.69±1.11 (3-7)	0.559
PII	1.04±0.43 (0-2)	1.31±0.43 (1-2)	1.23±0.44 (0.5-2)	1.19±0.43 (0.5-2)	0.019
*P-values correspond to Friedman tests for comparison of responses at successive time points.					

For the PlI, the Friedman test revealed significant differences between the time points analysed (P=0.019). Post-hoc Conover tests showed that these differences are due to the fact that the mean PlI at baseline was significantly lower than at later time points. Furthermore, the PlI at 6 months also shows a significant decrease as compared with 2 months (P<0.05 in each case) (Table I.5). The prevalence of BOP at the four analysed time points is shown in Fig. I.22.

Table I.5. Results (P-values) of post hoc comparisons between PlI and BOP values at baseline and after 2, 4, and 6 months of orthodontic treatment

Items	Baseline	2 months	4 months
PII			
2 months	$<10^{-5}$	-	-
4 months	<0.001	0.165	-
6 months	0.021	0.021	0.241
BOP			
2 months	0.063	-	-
4 months	0.023	0.824	-
6 months	0.023	0.562	1

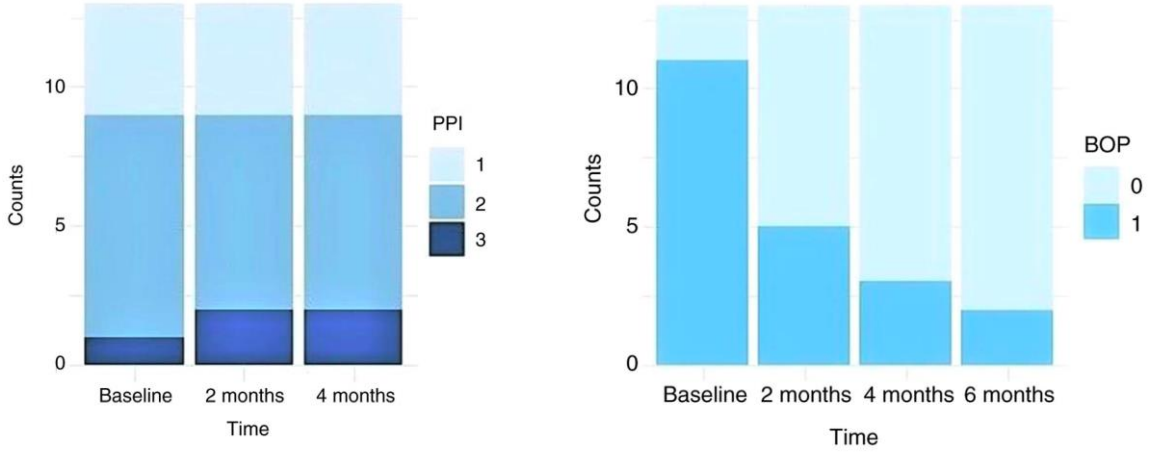


Figure I.22. Distribution of PPI and BOP at baseline, 2 and 4 months of orthodontic treatment.

The Cochran Q test showed that there are significant differences between the proportions at various time points (P=0.001). Post-hoc comparisons indicate that significant differences occurred between baseline and after 4 and 6 months **(**Fig. I.22). BOP frequency of occurrence decreased from 84.61 to 38.46%, 23.08, and 15.38% at 2, 4, and 6 months after orthodontic treatment, respectively (Table I.5). Assessing the presence of the periopathogens

Aa, Pg, Pi, and *Tf,* the Wilcoxon signed-rank test did not reveal statistically significant differences between the values recorded at baseline and after 6 months of treatment. For *Td*, the only periopathogen that exhibited an increase throughout the observation period, the differences were only marginally significant (P<0.1) (Table I.6).

Table I.6. Comparative detection scores at baseline and after 6 months of orthodontic treatment for periopathogens *Aa, Pg, Pi, Tf,* and *Td,* and P-values for the corresponding Wilcoxon signed-rank tests.

Detection score	*Aa*		*Pg*		*Pi*		*Tf*		*Td*	
	Baseline	6 months	Baseline	6 months	Baseline	6 months	Baseline	6 months	Baseline	6 months
Median	0	0	0	0	0	0	0	1	0	1
Range (min-max)	0-4	0-4	0-1	0-4	0-2	0-2	0-3	0-4	0-1	0-2
p-value	0.1814		0.3447		0.8241		0.7252		0.0649	

Discussion

To our knowledge, this is the first clinical study to describe at site-level the evolution of clinical and microbiological parameters in orthodontic patients previously treated for severe periodontitis.

Naranjo et al. (2006) noted a shift in the microflora populating the subgingival plaque after orthodontic bracket placement as well as a considerable increase of gingivitis in the test group. Another study found that levels of *Pg, Pi, P. nigrescens, Tf,* and *Fusobacterium spp.* increased after bracket placement in treated patients when compared with patients in the untreated control group. Super-infectious microorganisms such as *Enterobacter cloacae, Klebsiella oxytoca, Klebsiella pneumoniae,* and *Serratia marcescens* were detected by the authors in the treated group (Lauritano & Caccianiga, 2013). Surprisingly, a microbiologic study from 2004 attributed the marked improvement in the periodontopathogenic bacterial spectrum under fixed appliance therapy with metal brackets, NiTi arch wires, and stainless steel wires to metal corrosion, which entailed the release of nickel ions that are thought to be exclusively toxic to periopathogenic bacteria (Speer et al., 2004).

In the present study, clinical and microbiological evaluations were performed on each selected tooth at the site with the deepest residual PD (≥3 mm) to allow for potential changes of the respective surrogate parameters. All analysed sites were chosen on the aspect of the root that underwent compression so that GCF sampling and analysis could also include inflammatory resorption activity on the alveolar bone induced by the orthodontic movements (Bennett & McLaughlin, 2014).

The Friedman test for the mean values of PD, REC, and CAL variations did not indicate changes between the time points. The evolution of individual PDs revealed that, at 2 months, all 13 patients scored values equal to or lower than baseline.

The main periodontal objective of orthodontic treatment in patients with treated periodontitis is to maintain or improve attachment level. In our study, we concluded that PD did not change significantly following orthodontic movement, demonstrating that orthodontic treatment does not adversely affect the periodontal condition in patients with a history of periodontal disease. The results obtained are consistent with other clinical studies in the literature. It was previously shown that orthodontic treatment in patients with treated periodontal disease resulted in a 0.7 mm decrease in PD after one year of treatment (Ghezzi et al., 2008). Other authors, in a similar clinical trial, concluded that orthodontic treatment has no negative impact on PD variations. Although both studies used regenerative surgical techniques, the effects of orthodontic treatment on PD between the beginning of therapy and at the end of the assessment period are relevant (Cardaropoli et al., 2006). Other studies have

reported significant reductions in PD values following orthodontic treatment, concluding that orthodontic treatment can actually improve periodontal conditions (Passanzaei, 2007). Differently to the aforementioned studies, in our study the reduced magnitude of the PD changes could be potentially attributed to the severity of the treated periodontitis.

REC and PD did not exhibit statistically significant changes at the time points analysed. The evolution of REC at successive time points suggests that no change in the parameter occurred during the first 2 months interval. After 4 months of orthodontic treatment, there was a slight increase in mean values, while at 6 months there was a decrease. One important observation is that for 7 out of 13 patients, REC values did not undergo any change during the study period. One patient experienced a 1 mm increase over the 4 months interval that remained constant until the end, and two patients experienced a 1 mm reduction in REC over the 6 month period. The maximum recorded value was 3 mm for the first three intervals, and remained unchanged until the end of the assessment period. Similar to PD, a slight reduction in mean REC was observed in our study. These results provide some evidence for possible beneficial effects on periodontal status following orthodontic treatment. In the literature, the incidence of gingival recession following orthodontic therapy is disputed and the results are largely contradictory (Joss-Vassalli et al., 2010).

In our study, the mean CAL did not yield statistically significant variations (P=0.559). However, a 0.2 mm gingival attachment gain was noted at the end of the assessment period, when compared with baseline. These results are consistent with other clinical studies that did not identify statistically significant differences (Bennett & McLaughlin, 2014) or found no differences in CAL following orthodontic treatment (Gomes et al., 2007).

The only clinical parameter that recorded statistically significant differences was BOP. Its incidence was 90% at baseline, 40% at 2 months, and 20% at 4 and 6 months of orthodontic treatment. These results are somewhat surprising, given the positive relationship between gingival inflammation and the occurrence of BOP, as well as the pro-inflammatory effect of the orthodontic appliances on the gums. However, BOP, as a clinical symptom of inflammation, is largely influenced by plenty of other host-related and external parameters than the presence of periopathogenic bacteria and subgingival calculus deposits (Checchi et al., 2009).

Although PlI showed a significant increase between baseline and two months, reaching the maximum mean value of 1.31±0.43, it recorded a subsequent continuous decrease until the six months time point (all differences were statistically significant, P=0.019), reaching 1.19±0.43. Initial growth can be explained by the deterioration of oral hygiene due to the insertion of the orthodontic appliance, followed by a continuous decrease, possibly due to continuous re-enforced instruction in oral hygiene that was specific for patients undergoing supportive periodontal therapy. Generally, these scores indicate good oral hygiene throughout the study interval. However, there is no clear correlation between the evolution of this parameter and the BOP. While BOP scores exhibited statistically significant decreases between baseline and 2 months, PlI increased statistically significantly over the same interval and was the second largest change in this parameter over the observation period. The reduction in BOP could also be explained by the particular supportive therapy program, including monthly visits with a particular emphasis on preventing plaque accumulation in both supragingival and subgingival areas.

The results from the present study related to the evolution of BOP, PD, and PlI values are in line with those found in a study from 2009 (Checchi et al., 2009), which focused on the correlation of clinical parameters with the presence of subgingival plaque deposits identified by endoscopy. The results demonstrated a linear correlation between the presence of subgingival plaque and the proportional increase of BOP and PD. As in the present study, the authors concluded that differences in the efficacy of oral hygiene among patients make this

correlation difficult, and failed to find a concrete link between PlI and the evolution of BOP.

The variations in frequency of detection of the main periopathogens *Aa, Pg, Pi,* and *Tf* did not reveal statistically significant differences between the mean values recorded at baseline and those at six months of orthodontic treatment. The only periopathogen more frequently detected at the end of the observation period was *Td*, but was only marginally significant (P=0.0649). When analysing the comparative detection scores at baseline and after 6 months of treatment, a very low but detectable presence of *Aa*, *Pg,* and *Pi* was observed, with the median being 0 both at baseline and after six months of treatment. Although the median was 1 at baseline for *Tf*, the value remained unchanged after 6 months of orthodontic treatment. Even in the case of *Td*, which exhibited a slight increase at the 6 months time point as compared with baseline, the highest score was 2, the lowest score to ascertain detection.

These results demonstrate that the level of subgingival pathogenic bacteria was very low, close to zero, during the first months of orthodontic treatment. The low levels of detection correlate with the results obtained for clinical parameters, especially for BOP, demonstrating once again the effectiveness of systematic periodontal therapy prior to orthodontic therapy, and the role of properly performed oral hygiene during the maintenance phase. Similar results were obtained in a study on the incidence of *Aa* and *Pg* during orthodontic treatment with lingual brackets (Demling et al., 2009) and in earlier study of *Aa, Tf,* and *Pi* (Sargolzaie et al., 2013). The presence of the main periopathogens in the gingival sulcus during classical orthodontic treatment was investigated as well (Kim & Amar, 2006). In this study, *Aa, Pg, Pi,* and *Td* did not show statistically significant differences between baseline and the endpoint; *Td* exhibited only marginally significant differences. Thus, the authors concluded that patients with healthy periodontium prior to orthodontic treatment have decreased risk of further periodontal deterioration during orthodontic treatment. Although these results are not in line with the data from our present study, they show that orthodontic treatment does not have a negative effect on microbial flora as long as oral hygiene is properly carried out and the level of bacterial plaque accumulation is reduced to a minimum. Nevertheless, other studies have reported significant increases in pathogenic bacteria during orthodontic treatment (Ristic et al., 2007; Khan & Antony, 2013).

Although in the mentioned studies the detectable amount of periopathogens during orthodontic treatment increased significantly, the changes did not appear to have negative clinical effects; they returned to baseline with the removal of orthodontic appliances. Of note, studies that reported increased levels of periopathogens at the end of the treatment also showed significantly higher frequencies of detection at baseline, in contrast to studies that did not report significant differences and where the frequency of detection was absent or slightly positive. Thus, it can be argued that orthodontic treatment could have a negative effect on patients with high levels of bacteria before insertion of orthodontic devices, but does not cause them to occur in a healthy periodontium. Orthodontic treatment had no detrimental effect on the clinical parameters studied; however, we cannot draw a conclusion that there were improvements in periodontal conditions following orthodontic treatment, despite several studies that reported such outcomes (Re et al., 2003; Amiri-Jezeh et al., 2004).

One limitation of the present study is the relatively small number of investigated patients. This can be explained by the high number of inclusion criteria (both for the subjects and for the experimental sites), and by the numerous time points at which evaluations occurred. On the contrary, similar studies in the literature employed groups with a similar or a lower number of patients (Speer et al., 2004; Ghezzi et al., 2008). As in the daily practice the periodontal status of orthodontic patients previously treated for severe periodontitis is being routinely monitored using articulated supportive therapy sessions, there is clear need for further clinical studies to identify the periodontal sites at risk of deterioration and the measures necessary to mitigate the recurrence of the disease.

Conclusions

Within the limits of the present study, we concluded that there were no significant changes in the clinical parameters and microflora during the initial phase of orthodontic treatment in patients with periodontal support reduced by severe periodontitis, once the primary disease is systematically treated and the residual inflammation controlled. By correlating the clinical parameters with the microbiological ones, we inferred that residual levels of periopathogens did not negatively influence the periodontal health during orthodontic treatment in adult patients who underwent therapy for severe periodontitis.

I.5.2 Salivary metalloproteinase-8 and metalloproteinase-9 evaluation in patients undergoing fixed orthodontic treatment before and after periodontal therapy

Aim of the study

The aim of the study was to investigate the changes in the levels of matrix metalloproteinase-8 and matrix metalloproteinase-9 before and during orthodontic treatment and also after periodontal treatment and analysed their correlation with the bleeding on probing index (BOP). Furthermore, we have identified markers that could be used to investigate the periodontal status of orthodontic patients and to emphasize the need for regular periodontal maintenance during orthodontic treatment.

Materials and method

A research was conducted on 111 patients aged between 18 and 39 years, with a mean age of 25.5 - 5.4 years. All patients who were recruited completed the study. We included patients in generally good health who were about to receive fixed-appliance treatment and had a healthy periodontal status. The exclusion criteria were diagnosis of periodontal disease or a history of treatment, immune disease, systemic disease, smoking, pregnancy, lactation, and use of any medication that could interfere with OTM (antihistamines, cortisone, and hormones) within three months preceding the beginning of the study and use of antibiotics in the last six months. All patients received oral hygiene instructions prior to the beginning of the study. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

We determined the BOP index and the levels of MMP-8 and MMP-9 before placing the orthodontic fixed appliance (T1), one week after appliance placement (T2), and during orthodontic treatment, one month after applying the periodontal non-surgical treatment (T3). Orthodontic treatment was performed with a Roth prescription 0.022-in bracket slot appliance, which was bonded to the maxillary or mandibular arch. The first archwire was a 0.012-in nickel-titanium conventional wire.

The periodontal treatment aimed to eliminate supragingival and subgingival plaque and calculus. This was accomplished by comprehensive scaling and professional brushing using ultrasonic instruments (Hu-Friedy, Symmetry IQ® 3000, Chicago, IL, USA) and Gracey curettes (Hu-Friedy, Chicago, IL, USA).

Saliva sample collection was performed during routine appointments. This procedure was performed before any other clinical procedure in order to avoid blood contamination. Unstimulated whole saliva (3–4 mL) was collected in the morning from all participants (the subjects were instructed to skip oral hygiene that morning), at approximately 10 AM “a jeun” by instructing the patients to passively drool in a sterile polypropylene tube which was immediately frozen in a dry ice bath and stored at 80^{o}C until biomarker assessment. Unstimulated saliva was collected before placement of orthodontic appliances (T1), after the placement of orthodontic appliances but before periodontal therapy (T2), and during orthodontic treatment, one month after applying the periodontal treatment (T3).

Saliva samples were centrifuged at 13,000 rpm for 1 min at 4°C to remove cellular and insoluble debris. The supernatant was then transferred in a new Eppendorf 1.5 mL tube and appropriately labelled. Following the pre-processing steps, all samples were kept at −80°C until analysis.

For MMP analyses, we used the Human MMP-8 (Matrix Metalloproteinase 8) ELISA Kit and Human MMP-9 (Matrix Metalloproteinase 9) ELISA Kit from Elabscience, China. Statistical analysis was conducted using SPSS 25.0 (SPSS® version 25 for Windows®, SPSS Inc./IBM Group, Armonk, NY, USA) software, and $p < 0.05$ was considered to indicate a statistically significant difference.

Results

We analysed the salivary MMP-8 and MMP-9 levels before orthodontic treatment (T1), one week after orthodontic appliance placement (T2), and during orthodontic treatment, one month after applying the periodontal treatment (T3), as described in the materials and methods. For salivary MMP-8 levels, the highest values were recorded at T2, with a mean value of 0.267±0.20 ng/mL, while the lowest values were recorded at T1, with a mean value of 0.10±0.07 ng/mL (Table I.7).

Table I.7. Summarized levels of Metalloproteinase-8 (MMP-8), metalloproteinase-9 (MMP-9), and bleeding on probing (BOP) before orthodontic treatment (T1), one week after orthodontic appliance placement (T2), and during orthodontic treatment, one month after applying the periodontal treatment (T3).

Parameter	Mean (±Standard Deviation)
MMP9(T1) MMP9(T2) MMP9(T3)	0.450 ± (0.48) ng/mL 1.899 ± (1.82) ng/mL 0.100 ± (0.07) ng/mL *#
MMP8(T1) MMP8(T2) MMP8(T3)	0.100 ± (0.07) ng/mL 0.267 ± (0.20) ng/mL 0.140 ± (0.08) ng/mL *#
BOP(T1) BOP(T2) BOP(T3)	5.088 ± (2.72)% 16.224 ± (8.84)% 8.761 ± (4.56)% **##
T1—before orthodontic treatment; T2—one week after orthodontic appliance placement; T3—one month after combined orthodontic-periodontal treatment; BOP—bleeding on probing; MMP8—matrix metalloproteinase-8; MMP9—matrix metalloproteinase-9. *: significant difference compared to T2 (*: $p < 0.01$), using Wilcoxon Signed Ranks Test. #: significant different compared to T1 (#: $p < 0.01$), using Wilcoxon Signed Ranks Test. **: significant difference compared to T2 (**: $p < 0.01$), using Paired Sample T-test. ##: significant different compared to T1 (##: $p < 0.01$), using Paired Sample T-test.	

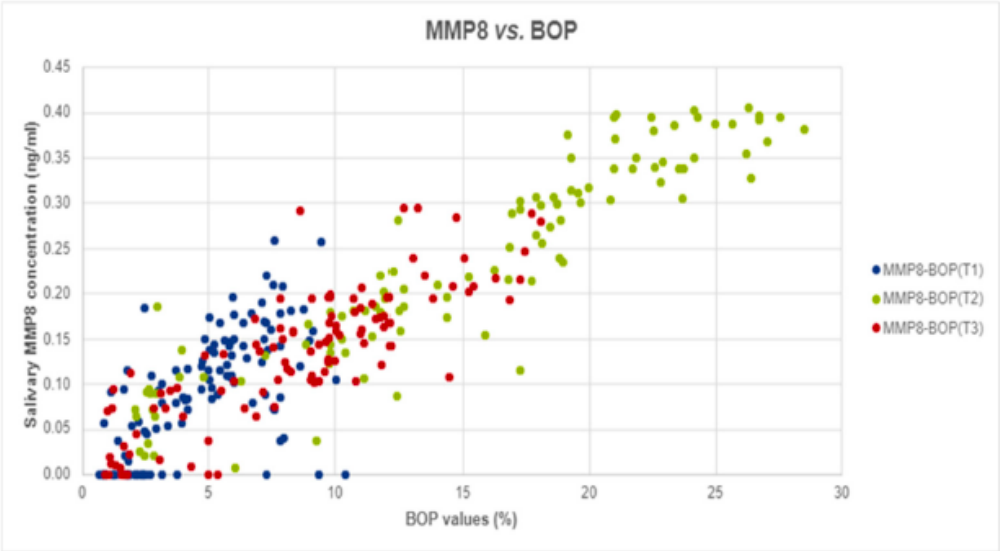


Figure I.23. The correlation between salivary MMP-8 and BOP values in T1, T2, and T3

After evaluating and comparing the values, we found that the mean value of MMP-8 at T3 was significantly lower than at T2 (p-value < 0.01) and significantly higher than that at T1 (p-value < 0.01) (Figure I.23, Table I.7).

For salivary MMP-9 levels, the highest values were also observed at T2, with a mean of 1.89 ± 1.82 ng/mL and the lowest value was observed at T1, with a mean of 0.45 ± 0.48 ng/mL (Table I.7), also in Figure I.24, we can observe a similar pattern to MMP-8. The mean MMP-9 level at T3 was significantly lower than that at T2 (p-value < 0.01), but significantly higher than that at T1 (p-value < 0.01). However, salivary MMP-8 and MMP-9 levels displayed a significant moderate correlation at T3 (Spearman's rho = 0.440, p -value < 0.01) and at T2 (Spearman's rho = 0.239, p -value < 0.05) (Figure I.24).

Similarly, the BOP values were assessed before placing the orthodontic fixed appliances (T1), one week after appliance placement (T2), and one month after periodontal treatment in this group of patients undergoing orthodontic treatment (T3). The highest BOP values were measured at T2, with a mean of 16.22%±8.84%, while the lowest BOP values were registered at T1, with a mean of 5.08% 2.72% (Table 1). The values of BOP at T2 were significantly higher than those at T3 (p-value < 0.01) and BOP values at T3 were significantly higher than those at T1 (p-value < 0.01) (Table I.7).

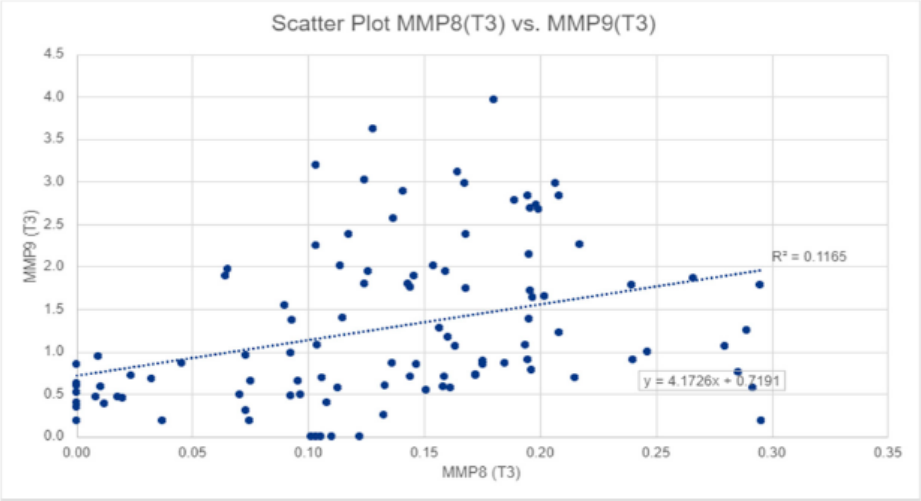


Figure I.24. The correlation between salivary MMP-8 and MMP-9 values in T3.

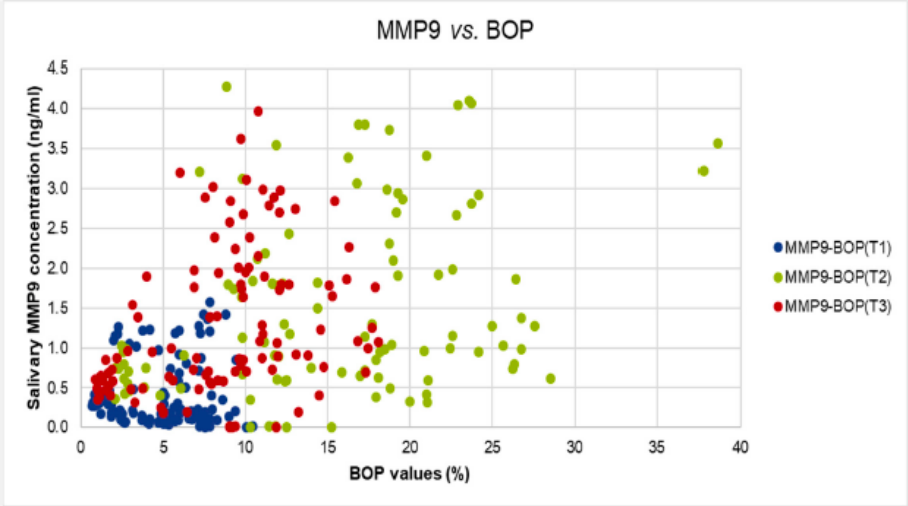


Figure I.25. The correlation between salivary MMP-9 and BOP values in T1, T2, and T3

Spearman Correlation analyses was performed to assess the potential correlation between MMP-8 levels and BOP. In our analyses we found strong, positive, and significant correlations at T2 (Spearman's rho = 0.939, p-value < 0.001) and T3 (r = 0.842, p-value < 0.001) and medium, positive, and significant correlation at T1 (Spearman's rho = 0.614, p-value < 0.001), as shown in Figure I.23.

We did identify also significant but moderate correlation between MMP-9 levels and BOP values at T2, and T3 (T2: Spearman's rho = 0.314, p-value < 0.01, T3: Spearman's rho = 0.426, p-value < 0.001), as shown in Figure I.25.

As anticipated, compared to the healthy group measurements for all three sampling times (T1, T2, T3), the localized gingivitis group showed higher values for BOP, MMP-8, and MMP-9 compared with the healthy group and the generalized gingivitis group showed higher values for all three markers than the localized gingivitis group (Table I.8).

Table I.8. Characteristics of study measurements among healthy, localized gingivitis and generalized gingivitis groups.

Parameter	Healthy group (N=201)	Localized gingivitis group (N=127)	Generalized gingivitis group (N=5)
MMP-9 (ng/mL)	0.843 ± 1.11	1.842 ± 1.58**	3.532 ± 0.50##**
MMP-8 (ng/mL)	0.098 ± 0.06	0.240 ± 0.09**	0.994 ± 0.26##**
BOP (%)	5.259 ± 2.80	16.411 ± 5.06**	39.366 ± 1.17##**
BOP—bleeding on probing; MMP-8—matrix metalloproteinase-8; MMP-9—matrix metalloproteinase-9. *: significant different compared to healthy group (**: p < 0.01). #: significant different compared to gingivitis group (##: p < 0.01).			

Mann-Whitney U test was performed to investigate differences among the three groups, the localized gingivitis group showed significantly higher levels of MMP-8, MMP-9 compared with the healthy group, the same results also comparing the markers from the localized gingivitis group versus generalized gingivitis group.

Table I.9. Results from ROC analysis of individual salivary biomarker levels of MMP-8 and MMP-9 comparing the healthy group to the localized gingivitis group.

Group	Optimal Cut-Off	Sensitivity	Specificity	FP	FN	AUC
MMP-8 (Healthy versus Localized Gingivitis)	0.152 ng/mL	0.898	0.811	0.189	0.102	0.924
MMP-9 (Healthy versus Localized Gingivitis)	0.874 ng/mL	0.732	0.697	0.303	0.268	0.752

We conducted ROC analysis in order to determine a cut-off for MMP-8, between healthy (BOP < 10%) versus localized gingivitis group (BOP < 10% and BOP < 30%). The results highlight an optimal cut-off, using the Youden index method, of 0.152 ng/mL for which we have a sensitivity of 89.8% and a false positive of 18.9% (Table I.9, Figure I.26).

Results from ROC analysis of salivary biomarker levels of MMP-8 comparing the localized gingivitis group (BOP 10% and BOP 30%) to the generalized gingivitis (BOP > 30%) group and the healthy group (BOP < 10%) to the generalized gingivitis group (BOP > 30%) resulted in an optimal cut-off of 0.420 ng/mL, respectively 0.491 ng/mL. These results are not statistically significant because in the group of patients with generalized gingivitis there were only five patients.

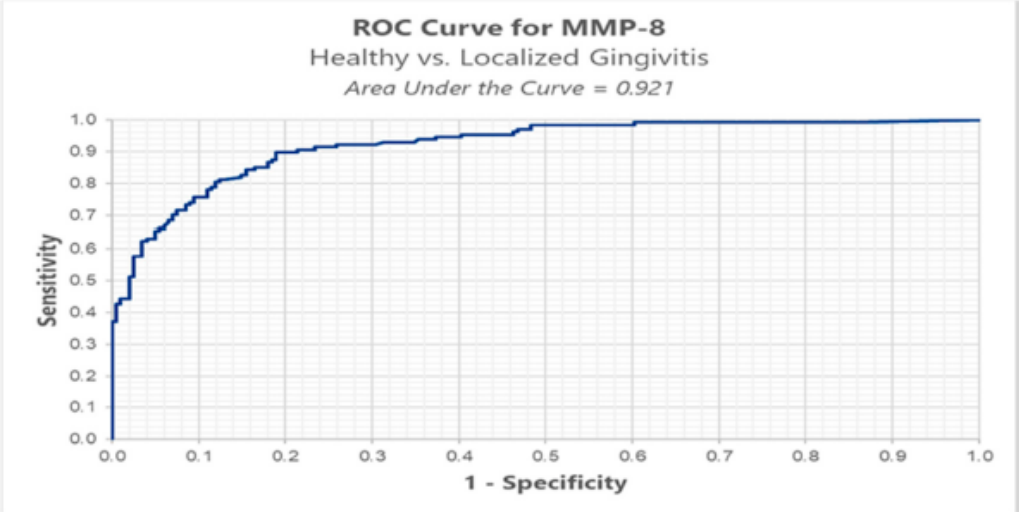


Figure I.26. ROC analysis of MMP-8 in healthy versus localized gingivitis.

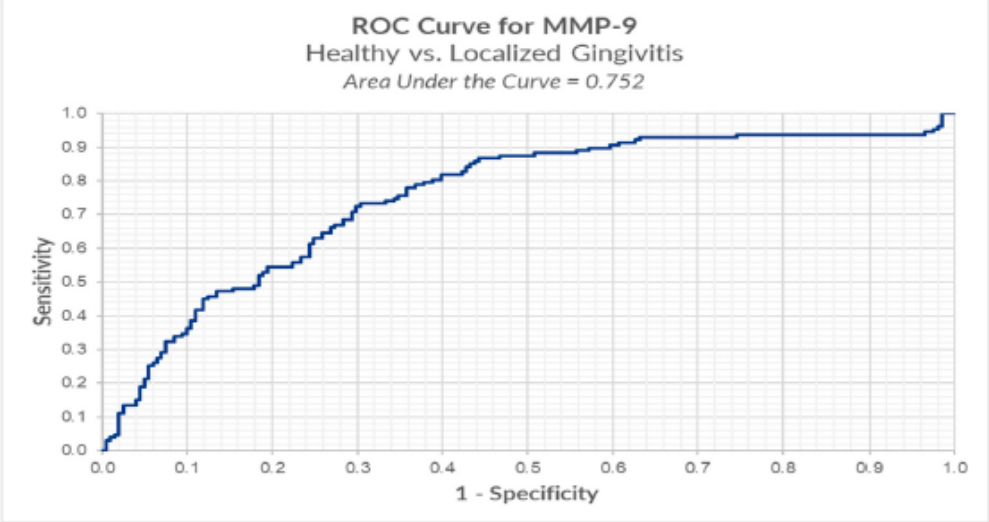


Figure I.27. ROC analysis of MMP-9 in healthy versus localized gingivitis.

Results from ROC analysis of salivary biomarker levels of MMP-9 comparing localized gingivitis group to generalized gingivitis group and healthy group to generalized gingivitis group resulted in an optimal cut of 0.491 ng/mL, respectively 2.923 ng/mL. These results are not statistically significant because in the group of patients with generalized gingivitis there were only five patients (Figure I.27).

Discussion

The findings of this study suggest that patients undergoing orthodontic treatment show a significant increase in BOP and MMP-8 and MMP-9 levels one week after orthodontic appliance placement (T2) and a decrease in these parameters one month after periodontal treatment (T3). Statistically significant correlations were found between MMP-8 levels and BOP values at T1, T2, and T3. Metalloproteinase-8 levels increased in inflammatory status, since it is the primary interstitial collagenase under inflammatory conditions, as stated by Ingman et al. (2005). This could explain the strong positive correlation found in our study between MMP-8 levels and BOP values.

Periodontal complications are one of the most frequent adverse effects of orthodontic treatment, and they include gingivitis, periodontitis, gingival recession or hypertrophy, alveolar bone loss, dehiscence, fenestrations, interdental folds, and dark triangles (Preoteasa et al., 2012). In addition, some researchers have shown clinical and microbiological changes

in patients undergoing orthodontic treatment that partially normalize after the removal of the appliances (Van Gastel et al., 2011). The assumption that long-term fixed appliances may lead to undesirable, but inevitable, qualitative changes in subgingival bacterial biofilms that gradually become periodontopathogenic over time is illustrated by previous studies (Perinetti et al., 2004).

Trombelli et al. (2018) show that gingival inflammation can be properly and easily detected and assessed using BOP. Its absence is a good indicator for periodontal stability, so it has the ability to reflect the periodontal status and the severity of inflammation. Although useful for scientific purposes, the BOP approach presents some disadvantages, such as the amount of time necessary for the quantitative analysis and the difficulty in distinguishing differences in the evaluation scale during a regular, thorough periodontal examination (Daly et al., 2001).

Nonetheless, oral fluid biomarkers exhibit the ability to provide further, more accurate insight when compared to regular clinical investigations. Moreover, those investigations (BOP, plaque index, probing depth, clinical attachment level, and radiographic recordings) illustrate only retrospective data, and not the current disease status (Buduneli & Kinane, 2011).

In light of these factors, identification of a specific biomarker for assessing periodontal status during OTM is important. This is necessary since completion of orthodontic treatment without effects on the periodontium is essential but challenging. One should also consider the frequent iatrogenic effects caused by orthodontic treatment; some authors agree that preventive measures must be considered for all patients undergoing orthodontic therapy (Bardal et al., 2011).

Various studies have shown that increased MMP-8 and MMP-9 levels characterize not only periodontal disease (Sorsa et al., 2006; 2016) but also tend to increase during OTM (Xu et al., 2020). Our study evaluated MMP-8 and MMP-9 levels and BOP at T1, T2, and T3 and identified a significant positive correlation between the MMP-8 levels and BOP before and after periodontal treatment. Indeed, these findings are in agreement with the results of other studies in which MMP-8 levels were highly correlated with BOP (Bosca et al., 2012). Furthermore, we observed a medium positive statistically significant correlation between MMP-9 and BOP values before and after orthodontic treatment and periodontal treatment.

In our study, we conducted ROC analysis in order to determine a cut-off for MMP-8 andMMP-9 between healthy versus localized gingivitis group versus generalized gingivitis. Results from the ROC analysis of salivary biomarker levels ofMMP-8 andMMP-9 comparing healthy versus localized gingivitis resulted in an optimal cut-off of 0.152 ng/mL and respectively 0.874 ng/mL. This is the first study to analyse such a value in orthodontic patients and we believe it is a valuable tool that can assess the current periodontal status and prognosis of a patient and can be further studied in patients with more severe periodontal disease. Thus, we propose the use ofMMP-9 and especiallyMMP-8 levels as biomarkers of periodontal disease during orthodontic treatment to facilitate the detection of early periodontitis or gingivitis. Although BOP andMMP-8 levels have been shown to allow distinction between a healthy periodontal status and gingivitis or periodontitis cases (Bosca et al., 2012), other studies have shown conflicting or contrary results (Gursoy et al., 2010; Kushlinskii et al., 2011). MMP-8 is associated with the diagnosis of periodontal disease (De Morais et al., 2018), the severity of periodontal inflammation, evolution, and follow-up of therapy (Bosca et al., 2012). It can also be used to monitor periodontal disease status (Sexton et al., 2011). Therefore, these biomarkers can be used to identify the inflammatory status of patients undergoing orthodontic treatment and to measure results after periodontal treatment.

The major component of the periodontal extracellular matrix is collagen type I.MMP-8 levels have been shown to be correlated with collagen type I degradation products,

overcoming the protective mechanism of MMP tissue inhibitors in active disease sites as opposed to inactive sites in patients with periodontitis and healthy controls (Sorsa et al., 2016). MMP-8 is the key collagenolytic component found in the gingival tissue and oral fluids (Hernandez-Rios et al., 2016). Therefore, MMP-8 is considered a biomarker in periodontitis. This could explain its significant and strong correlation with BOP. A recent study by Shirozaki et al. (2020) found that the percentage of sites with BOP increased after orthodontic therapy, as our data also confirms.

In our study, salivary MMP-8 levels in patients undergoing both orthodontic and periodontal treatment were 0.5-fold smaller than those before applying periodontal treatment, which is in agreement with the study performed on the gingival crevicular fluid of patients with no orthodontic appliances by Mäntylä et al. (2003). Interestingly, Marcaccini et al. (2009) found strong correlations between the plasma levels of MMP-8 and MMP-9 before and after periodontal treatment in patients without orthodontic appliances. Thus, further studies with larger groups of cases might clarify any potential links among MMP-8, MMP-9, and BOP before periodontal treatment (in the current study with only 111 subjects, the p - value for the correlation between MMP-9 and BOP was <0.01). Since we only aimed to evaluate the local inflammation status using biomarkers such as salivary MMP-8 and MMP-9 values and BOP percentages, we conceived the study without including any other clinical measurements.

This is the first study to evaluate salivary biomarkers in patients undergoing orthodontic treatment before and after periodontal therapy. A biomarker is easy to assess, takes less chair time, and documents the current inflammatory status. Here, we propose that the MMP-8 level combined with BOP values could be analysed as a biomarker before and during orthodontic treatment in order to identify the individual periodontal inflammatory status and disease prognosis.

Nevertheless, the study had some limitations. The sample size was small, and evaluation of data after three, six, and 12 months or at each month during the first six months would have yielded more applicable results. Future studies could include an assessment of each patient’s measures of hygiene (by means of questionnaires or by plaque index evaluations) in order to identify more specific correlations between results and the used hygiene methods.

Conclusions

In our study patients undergoing orthodontic treatment show a significant increase in BOP, MMP-8, and MMP-9 levels one week after orthodontic appliance placement and a decrease in these parameters one month after periodontal treatment. Strong positive statistically significant correlations were found between MMP-8 levels and BOP and medium positive statistically significant correlations between MMP-9 and BOP values before and after orthodontic treatment and periodontal treatment. MMP-8, MMP-9, and BOP could be used to assess the periodontal status of orthodontic patients.

I.6 The complex diagnosis of systemically affected patient

State of the art in the relationship between periodontal disease and systemic conditions

The human body is a single unit made up of an infinite number of biological processes. They are so intertwined that the slightest anomaly in one of these processes can have profound effects in multiple regions of the body. Thus the link between the oral cavity and the general state can be stated by: "The cavity is the window of the health of our body". It can show signs of illnesses, general infections and nutritional deficiencies. This notion was introduced by William Hunter in 1990 in the medical literature with a report entitled "Oral

sepsis as a cause of illness". As an area infected with a pathogenic organism, the oral cavity is explored as a possible cause or exacerbating factor for certain systemic conditions.

This is why in recent years, particular attention has been paid to oral septicaemia and its relationship with certain diseases such as cardiovascular disease, diabetes, respiratory disorders, osteoporosis and adverse outcomes of pregnancy. The concept of “periodontal medicine” was born.

Periodontal disease can be defined as multifactorial infectious disease. The host response to infection is an important factor in determining the extent and severity of periodontal disease. Systemic factors modify periodontitis mainly through their effects on the immune and inflammatory mechanisms. Several factors can lead to an increase in the prevalence, incidence or severity of gingivitis and periodontitis. The effects of a large number of systemic diseases on periodontitis (and vice versa) are unclear and it is often difficult to establish a causal link between these diseases and periodontitis.

The dental specialist is part of the patient care journey, as a preventive and diagnostic actor. He must adopt an attitude of comprehensive medical care beyond the oral cavity.

Gingival and periodontal diseases are manifested at global level, in all populations subjected to investigations. The clinical signs of periodontal destruction may appear at any age, the data demonstrating the existence of populations resistant to periodontitis being extremely scarce. The periodontal disease is an inflammatory affection of destructive type, whose main factor in its etiology is, apart from other numerous favourizing local and general causes, the bacterial plaque. The periodontal disease may appear in various forms, over a large manifestation area, starting from tissular affection up to destruction of the periodontium, which, in certain cases, may lead even to tooth losses.

The periodontal pathology is characterized by gingivitis, periodontitis, periodontal manifestations caused by certain systemic problems, its manifestation and evolution varying for each form in part. The irritative, functional factors affect the general condition of the organism, modifying its defense capacity and, even if failing to initiate the destructive process, accelerate its progress and ratio of tissular destruction (Macovei-Surdu et al., 2013).

The bacterial plaque appears as the main etiological agent, however, the systemic and local factors capable of modifying the response of the periodontal tissues to plaque deposition may be identified by anamnesis and strict clinical examination (Mros & Berglundh, 2010). Numerous systemic factors of risk may modify the effect of the plaque upon the host. The environment and the genetic factors will influence the microbe-host equilibrium. Among the systemic factors that may influence the periodontal disease, mention should be made of cardiovascular affections, diabetes, endocrine disorders, obesity, metabolic diseases and even digestive and renal maladies (Bouchard, 2015).

Smoking represents a risk factor of both systemic and local nature, involving an extremely complex mixture, of over 4,000 substances which contain carbon monoxide, hydrogen cyanide, oxygen radicals, an increased number of carginogenic compounds and the main addicted molecule – nicotine (Solomon et al., 2012), rapidly absorbed at lung level. Administration of nicotine increases blood pressure, heart frequency, breathing frequency, while decreasing the temperature of the teguments, as a result of peripheric vasoconstriction. Nevertheless, in other sites, such as, for example, striated musculature, nicotine induces vasodilatation. The mechanisms of the adverse effects induced by smoking have been established, however, their exact molecular paths are still to be identified.

Age, sex, education level, the socio-economic condition, the systemic maladies, genetic predisposition represent systemic factors intervening in the occurrence and evolution of the periodontal disease. To all these, favourizing local factors may be associated, such as a scarce oral hygiene, the scale, edentations, malocclusions, parafunctions, or the incorrectly applied odontal, prosthetic, surgical, orthodontic treatments. Thus, to obtain a correct general image

of the periodontal status *versus* the systemic context and the local factors, as well as for a correct and complete approaching of the patient, all these data should be carefully collected, prior to the elaboration of an adequate and individualized treatment plan (Bouchard, 2015).

Inflammatory mechanisms in the relationship between periodontal disease and systemic conditions partially reside in the proinflammatory reactions which are the result of a complex interplay of bacterial inhabitants of subgingival biofilm and macrophages from the periodontal tissues (Miranda et al., 2019). The release of inflammatory mediators and bacterial factors from the periodontal tissues into the systemic circulation induces a systemic inflammatory burden which interferes with the function of insulin receptors and lead to peripheral insulin resistance (Chen et al., 2015).

Data regarding the deleterious effect of chronic periodontitis on glycemic control in T2DM is controversial. Outcomes of a meta-analysis and several studies revealed a significant improvement of glycemic control, quantified using HbA1c, after periodontal therapy (Wang et al., 2017). In contrast, some studies did not indicate a significant HbA1c reduction after periodontal therapy, the periodontal therapy failure to control the periodontal infection and associated inflammation being a potential explanation for the controversial reports (Engebretson & Kocher, 2013).

The association between these two chronic disease, diabetes and periodontitis is considered to be bidirectional. The mechanism involved in this association show that hyperglycaemia cause the formation of advanced glycation products, microvascular diabetic chronic complications with are related with changes in cytokines secretion profile (Mota et al., 2015).

The relationship between diabetes mellitus (DM) and periodontitis has been reported in numerous studies. About this relationship it can be said that diabetes is a risk factor for periodontitis and periodontitis may have a negative effect on glycaemic control. Also, periodontal disease is an important factor for increased risk of diabetic complications (Licardo et al., 2019). Although DM and periodontal disease are different medical entities, they have mutual influence by biochemical mechanisms, at the cellular and molecular levels.

The composition of gingival crevicular fluid (GCF) generally reflects the inflammatory condition of gingival and periodontal tissues, and for this reason it has been of great interest to periodontal researchers. The GCF analysis provides information specific to that periodontal site (Dogukan et al., 2018). It's known that the initial periodontal therapy changes GCF cytokine levels in patients with aggressive and moderate to severe forms of chronic periodontitis (Fokkema et al., 2003). This has been demonstrated by using an enzyme-linked immunosorbent assay (ELISA), showing that IL-12 increased after initial therapy, in contrast with IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF- α that have remained unchanged. In decompensated diabetic patients with poor control of glycaemic status the severity of periodontal disease in T2DM is growing. A study focused on the level of interleukins in saliva showed that the IL-17 level and probing pocket depth were not affected by glycaemic status (Gürsoy et al., 2015).

Basically, the function of inflammatory cells, such as neutrophils, macrophages and monocytes is altered in diabetic individuals, the severity of periodontitis being tightly correlated with the impairment of chemotaxis (Gurav & Jadhav, 2011). Patients with severe periodontitis exhibit a reduction in the chemotactic ability of neutrophils compared to those with mild periodontitis or non-diabetic individuals with severe or moderate periodontitis (Hegde & Awan, 2019). In addition to changes in phagocytic and chemotactic function, some studies on diabetic subjects and periodontal damage have revealed other changes in cells involved in mediating the immune-inflammatory response in this association, including intracellular destruction and adhesion ability. These defects in the body's immune system may predispose people with T1D to periodontal harm (Kumar et al., 2014).

Other studies also display cytokine alterations in the salivary /gingival fluid, and / or gingival tissue in individuals suffering of diabetes and periodontal injury compared to healthy subjects (Costa et al., 2010), although independent studies performed on the same subjects category, could not obtain the same evidence (Duarte et al., 2011).

Regarding the means by which periodontal disease could have a contribution in the pathogenesis of cardiovascular disease there are several possible mechanisms:

1. Release of endotoxins in the blood stream;
2. The effects of heat shock proteins;
3. Altered lipid profiles caused by periodontal pathogens which determine the oxidation and aggregation of low-density lipoprotein cholesterin;
4. Generation of acute-phase reactants such as CRP in the liver (Bhateja & Arora, 2014).

Most often, the initiation of CVD incidents is determined by the existence of atherosclerosis— accumulation of atheroma plaques in arteries due to endothelial damage which cause an immuno-inflammatory response. Escalation of atherosclerosis can cause thromboembolisms which may ultimately cause an infarction and/or stroke (Gimbrone Jr & García-Cardeña, 2016).

This ongoing low grade subclinical inflammation, caused by acute and chronic inflammatory conditions diseases, hinders the function of endothelial tissue and the mechanical properties of blood vessels (Taylor & Hennekens, 2018). Concurrently, MMP-8 is secreted by smooth muscle cells that exist at the junction of the intima and media layer of arteries, thus promoting myogenesis. The hyperplasia of these cells induces fibrosis and the subsequent establishment of a fibrous plaque. In the course of time, cholesterol accumulates on the plaque and forms lipid streaks that can block the blood vessel. As lipid structures accumulate in excess this undermines the compact structure of the plaque and instigates its rupture causing thrombus formation. This in turn discharges thrombotic factors that promote coagulation when it comes in contact with platelets and can determine a thromboembolism (Cowan et al., 2019).

Microbes frequently incriminated in the development of periodontal disease are more often than not gram-negative that promote increased bacteraemia after tooth brushing and routine dental procedures such as scaling, furthermore, periodontal pathogens can penetrate endothelial cells and lead to malfunctions (Solomon et al., 2015).

As a result of this, periodontal microbes and their deleterious products are discharged into the systemic circulation via the ulcerated sulcular epithelium of the gingival tissue becoming a source of systemic inflammation through the release of inflammatory mediators, which can promote insulin resistance and possibly offer an explanation for the perio-systemic link (Hasturk & Kantarci, 2015).

Type 1 T-helper (Th1) responses are induced by the previously mentioned antibodies and inflammatory cytokines and help mobilize monocytes/macrophages to the affected site where the oxidized LDLs are engulfed, thus constituting foam cells and further generating atherogenesis. In later stages foam cells go through a process of necrosis, releasing additional inflammatory stimuli, and through that creating the necrotic central area of advanced lesions (Kumar et al., 2014).

Regarding the effect that periodontal treatment has on the evolution of cardiovascular diseases, recent literature reports moderate long-term beneficial outcomes on CRP levels – a marker for systemic inflammation and clinical criteria of endothelium function. CRP continues to be in a firm position as a relevant marker of CVD risk (Merchant & Virani, 2017).

The clinical trials and meta-analyzes that have been performed, have demonstrated involvement in the host immune responses, of the balance between two categories of

cytokines, resulting in increased serum concentrations in patients belonging to the group with joint damage compared to the group of healthy subjects. These results allowed the hypothesis to be issued, according to which destructive changes and inflammatory processes occurring in the joints are generated and maintained by both subpopulations of cytokines (Boissier, 2011).

Either involving periodontal tissues or developing into chronic liver diseases, the holistic and integrated setup of the human body allows common influencing and impact. The immune response tries to solve and repair certain pathological situations, but in this process, it can also aggravate and deteriorate the status of other developing conditions. The periodontal and hepatic inflammatory reactions can have mutual influence and exacerbate one another, when existing in the same patient, by the synergetic action of the pro-inflammatory mediators (Han et al., 2016).

Chronic kidney disease (CKD) is a heterogeneous condition with the most serious adverse outcomes including debilitating metabolic complications of decreased GFR progressing to end stage renal disease (ESRD) and increased risk of cardiovascular disease (CVD). Epidemiologically, the most important risk factor for cardiovascular comorbidities of CKD is inflammation. The group of ESRD-patients with or without dialysis has high mortality rate, traditionally explained by the risk factors of atherosclerosis (diabetes, hypertension, dyslipidaemia). Lately there have been taken into consideration other factors contributing to the high rate of mortality such are inflammation, malnutrition and predisposition to infection (Alani et al., 2014; Subbiah et al., 2016).

In 2017 in Romania the first comprehensive study which aimed to explore the link between periodontal disease and quality of life was developed, assessed with the Short Form 36-Item Health Survey in haemodialysis patients (Sincar et al., 2017). The study showed a high prevalence and severity of periodontal disease, evaluated with the gingival and periodontal index. High scores of the gingival and periodontal components of the index were associated with low QoL, both on physical and on mental components in this group of patients.

CKD itself is a complex disease with numerous comorbidities all recognizing as a common link the inflammation. ESRD is treated either through dialysis or renal transplant. It is known that dialysis increases the inflammatory burden. The already challenged host, often associates periodontitis that increases even more the overall systemic inflammation. Cytokines seem to be the common link between pathophysiologic mechanisms of these diseases.

We conducted a study with the purpose to evaluate the periodontal status, together with the systemic and local risk factors, on a group of 170 patients, subjected to a minute clinical examination. The periodontal diagnosis was based on specialized and also on additional (*i.e.*, radiological: retro-dento-alveolar images and ortopantomographies, photographic images for documentation) examinations. Anamnesis of the patients provided important information on the grounds of the presentation, medical history of the patients, living and working conditions, possible vicious habits, while the dental clinical exam offered data on the odontal and periodontal status, and local factors favorizing the development of the periodontal disease. Out of the systemic maladies observed, an important part was held by cardiovascular diseases (20 cases, of which 13 with arterial hypertension). Also registered were 6 cases of digestive affections (gastritis - 2, gastric ulcer - 1, duodenal ulcer - 1, gastro-oesophagian reflux - 2), 4 cases with neurological diseases (2 with schizophrenia and 1 with epilepsy), 4 cases of diabetes (1 of type I and 3 of DZ type II). At the level of the experimental group, 42.94% of the patients were smokers (73 persons, among which 13 chronic smokers) and 17.05% were subjected to psycho-social stress.

Medical history may provide significant data, for both the therapeutical conduct and for the identification of the systemic risk factors. Development and advance of the periodontal

disease in a patient are" individualized" by a number of endogenous and exogenous factors. Evaluation, understanding and an adequate management of such factors facilitate prevention or control of the disease, when a certain periodontal affection is manifested. The bidirectional character between the periodontal disease and a series of systemic pathologies (cardiovascular diseases, diabetes mellitus, endocrine maladies, obesity etc.) is well-known. In the present study, an important part was held by the cardiovascular diseases (30 cases).

A series of environmental factors and some vicious habits may appear as important risk factors for the progress of the periodontal disease. One of them is stress. The stressing events characterizing the social and occupational environment showed their capacity of influencing the organism at the level of certain systems, such as the endocrine or immune system, thus inducing systemic modifications. Association of stress with other maladies is more powerful in the case of infectious, inflammatory pathologies, also affecting the healing processes (Broadbent et al., 2003). In the present study, a significant number of patients declared that they suffer from psycho-social or occupational stress (17.05%). The mechanisms through which the psycho-social stress may affect the periodontal status are complex, one of the suggestions put forward being that one of the plausible cause may involve behavioural modifications, leading to smoking and to a scarce oral hygiene.

An important number of subjects declared that they are smokers. The higher risk, in smokers, for periodontal lesions has been confirmed by numerous studies (Hanioka et al., 2019), the values recorded being four times higher than in the case of non-smokers. Such data suggest some dose-effect relation between the number of cigarettes smoked daily and the susceptibility to periodontitis. It is estimated that more than 40% of the cases of periodontitis in adults may be attributed to daily smoking. Clinically relevant is the observation that smoking interferes with healing of lesions after debridement (Naji et al., 20019), periodontal surgery and procedures of guided bone regeneration.

Crohn's disease is a chronic inflammatory pathology of the digestive system first described in 1932 by Dr. Burrill B. Crohn, gastroenterologist at Mont Sinai hospital in New York (Magali, 2016).

The inflammation can affect any part of the digestive tract, from the mouth to the anus. But most often it settles at the junction of the small intestine and the colon. However, there are frequently oral symptoms which can lead to screening for the disease when it is not known to the patient.

It is therefore imperative for the dental specialist to know this pathology and understand the consequences at the oral level to take care of the patient as a whole.

Conversely, when Crohn's disease is suspected in a subject, a systematic full oral examination may be important to confirm the diagnosis.

Subsequently, collaboration between the dental specialist and the gastroenterologist seems essential in order to set up an optimal treatment and protection of the oral mucous membranes.

The inflammatory response is an essential mechanism of the innate immune response. It manifests as swelling, redness, increased temperature, and pain associated with an influx of blood plasma to the affected site.

Granulocytes and blood monocytes which then transform into macrophages flow to the site in order to carry out phagocytosis to eliminate the attacking agent. So-called sentinel cells, such as dendritic cells for example, patrol the tissues and recognize the attacking agents. They then secrete mediators of inflammation that will cause the inflammatory reaction (Magali, 2016).

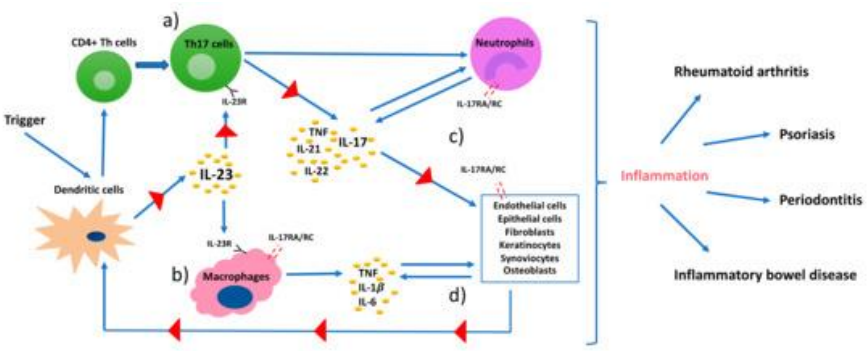


Figure I.28. The immune-mediated inflammatory diseases – pathophysiological model (Bunte & Beikler, 2019)

If the innate immunity is insufficient to fight the infectious agent, adaptive immunity is initiated by the dendritic cells which, after ingesting the infectious agent, present a fragment of it in the lymph node causing the production of antibodies and T cells specific to this agent (Lira & Figueredo, 2016) (Figure I.28).

Figueredo et al (2011) shown the higher concentration of interleukin 18 in the serum of patients with Crohn's disease and chronic periodontitis, on the other hand the concentration of this same molecule in the crevicular fluid is lower in these patients compared to healthy patients with periodontitis. This interleukin 18 regulates the immune response and the inflammatory response. Interleukin-4 is found in lower amounts in the crevicular fluid of patients with Crohn's disease and periodontal disease compared to patients with only periodontal disease. This cytokine allows the regulation of innate or acquired immunity, the differentiation of lymphocytes but also the production and secretion of collagen, except periodontitis is due to the destruction of collagen resulting in loss of attachment. The decrease in interleukin 4 could then have an effect on the increase in periodontal destruction, initiation and progression of periodontitis in patients affected by Crohn's disease.

A hypothesis has been proposed by Badran et al. (2015) regarding the role of microparticles, which are mediators of inflammation released by endothelial cells and platelets. They are physiologically present in the blood but their presence is increased in the case of Crohn's disease in particular. Badran and his team have suggested that periodontitis, by generating a constant increase in the level of microparticles locally and then passing into the bloodstream, may be partly responsible for the increased presence of certain systemic diseases in patients with periodontal disease, especially those with vascular dysfunction or with inflammatory deregulation like Crohn's disease. This would be similar to the passage of periodontal bacteria into the blood through contact between the biofilm and the epithelium of the ulcerated periodontal pocket.

Crohn's disease is constantly progressing in terms of epidemiology; therefore, it is important that this pathology and its consequences are known by the entire medical profession so that the management is as comprehensive as possible, including from an oral point of view. Periodontal disease, on the other hand, is constantly widespread in the general population and very often isolated, this is why the appearance of this disease will not systematically lead to the search for another systemic pathology. However, when diagnosed with Crohn's disease, several studies show that the risk of developing periodontal disease is increased. This is due to identical risk factors and to a fractional diet which leads to an increase in the formation of dental plaque. It would be interesting to determine whether the stabilization of the periodontal state allows to limit the occurrence of acute phases and the deterioration of the pathology in the patient with Crohn's disease.

I.6.1 The relationship between periodontal disease and cardiovascular diseases

State of the art in the relation CVD-periodontal disease

Cardiovascular disease is the number one killer worldwide. According to the WHO, cardiovascular disease (CVD) includes a number of disorders affecting the heart and blood vessels such as:

- High blood pressure (increased blood pressure);
- Coronary heart disease (heart attack or infarction);
- Cerebrovascular diseases (stroke);
- Peripheral arteriopathies;
- Heart failure;
- Rheumatic heart disease
- Congenital heart disease
- Cardiomyopathies.

A particular form of CVD, atherosclerosis, is characterized by the loss of elasticity of the arteries, due to sclerosis caused by the accumulation of fatty substances (lipids, essentially LDL cholesterol), in one of the three coats, constituting the wall of the arteries (the intima), and interesting above all, the large and medium arteries. Sclerosis constitutes plaques which can rupture and block a vessel, thus causing complications: myocardial infarction, ischemic stroke or arteritis of the lower limbs (Chistiakov et al., 2017).

Cardiovascular pathologies and periodontal disease are both chronic, multifactorial pathologies and have in common a number of modifiable and non-modifiable risk factors such as age, sex, socio-economic background, tobacco, obesity, diabetes and stress (Jeftha & Holmes, 2013). It is now recognized that periodontitis is at increased risk for CVD (Yu et al., 2015). The authors have highlighted a relationship between arterial involvement and oral bacteraemia. There are therefore two hypotheses:

- An indirect immuno-inflammatory reaction which would be induced by the increased secretion of pro-inflammatory markers / mediators: certain cytokines IL-1, IL-6, TNF-α, CRP and oxidative stress involved in atherogenesis. These cytokines have been observed in higher concentrations in patients suffering from periodontal disease and in decrease in the treated subjects.
- A direct bacterial reaction (on the target organ). There is a positive association between antibody levels and the presence of coronary heart disease in large population-based cohorts (Han et al., 2014). Periodontopathogens are grafted to a localized vascular lesion and promote the recruitment of macrophages which participate in atherogenesis. Thus they participate in the development of the atheromatous lesion. According to the Damgaard et al. study (2017), circulating IgG antibody levels against Pg and Aa are correlated with loss of periodontal attachment and could be used as a biomarker of periodontitis and cardiovascular disease. However, only the association of anti-Pg antibody levels reaches a statistically exploitable significance after adjusting common risk factors (such as age). Obviously, it must also be taken into account that the periodontal biofilm can vary depending on the stage of the periodontal disease (Damgaard et al., 2017). Pg has been detected in human atherogenesis plaque, it is linked to atherosclerosis (Hasturk et al., 2012). In addition, the abundance of Fn is positively correlated with LDL cholesterol and total cholesterol (Koren et al., 2011).

According to the WHO, obesity is a chronic disease caused by an abnormal or excessive accumulation of fat which poses a health risk. Body mass index (BMI) applies to both genders and all age groups. It should however be considered as an approximate indication because it does not necessarily correspond to the same percentage of fat mass according to the individuals. BMI is not yet usable for children.

Obesity is a major global health problem since it represents a significant comorbidity of diabetes, cardiovascular disease, reproductive system dysfunction, osteoarthritis and multiple cancers. Many factors come into play: genetic, biological, social, behavioural factors. This problem mainly affects developed countries (Bouchard, 2015).

Despite this coexistence of several definitions which complicate its diagnosis, the metabolic syndrome is characterized by the conjunction of disorders, often moderate, of carbohydrate origin (high fasting glucose), lipid (high level of LDL triglyceride and low level of HDL cholesterol) or vascular (hypertension), associated with abdominal obesity and insulin resistance, which will act concomitantly, cause type 2 diabetes and predispose to atherosclerosis and therefore to cardiovascular diseases (Figure I.29). To present this syndrome it is necessary to have at least three of the associations mentioned above. Infection, inflammation and oxidative stress could mediate the associations described in the literature between periodontal disease and metabolic syndrome (Blasco-Baqué, 2013).

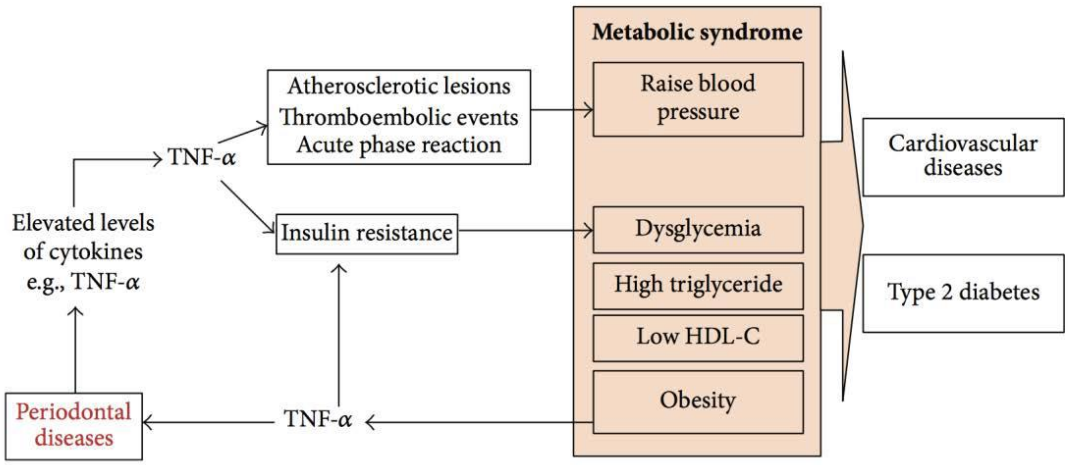


Figure I.29. A biological working model on the onset of periodontal disease in obesity and metabolic syndrome (Khosravi et al., 2013)

There are two mediators that are important to emphasize: TNF-α and IL-6. They are both at high levels in obesity and metabolic syndrome. These two pro-inflammatory mediators are found to be highly concentrated in the adipose tissue of obese patients, and therefore have a role in regulating insulin sensitivity.

TNF-α is a well-known mediator of periodontitis; it helps stimulate the formation of osteoclasts, they are early promoters of hosts with periodontal disease-causing bacteria (they trigger an immune response) and they regulate MMPs (breakdown of connective tissue). Adipocytes secrete TNF-α, which explains why excess fat leads to chronic systemic inflammation. Also, adipose tissue synthesizes other cytokines. According to some studies, there are high levels of TNF-α in the gingival tissue of obese patients.

The periodontal microbiota could have an influence (as with diabetes) on the metabolism of the host, influence its systemic inflammation and therefore act on insulin resistance. A diet high in fat would bring more Gram negative bacteria and change the gut microbiota. Indeed, periodontal disease is also characterized by its large number of Gram - anaerobic bacteria (Minty et al., 2019). The circulating LPS (inflammatory and antigenic molecules) of these bacteria create systemic inflammation.

Taken together, these data support the view that in obesity and metabolic syndrome, elevated levels of TNF-α and possibly IL-6 increase the risk of the development of periodontal disease directly through the mechanisms discussed previously and indirectly by triggering the host immune response induced by bacteria in obesity and metabolic syndrome.

Virto et al. (2018) demonstrated the comorbid effect of periodontitis and obesity at the periodontal and systemic level by demonstrating an increase in periodontal severity, increased systemic inflammation and deregulation metabolic affecting glucose metabolism and dyslipidaemia.

We conducted a study in order to assess the levels of cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL) and triglycerides on subjects with and without periodontal disease. In our study the degree of periodontal lesions was positively correlated to the cholesterol plasmatic levels. The pathological levels of the triglycerides were around 6.5 times more frequent in periodontitis patients than in control group subjects, while no HDL difference was observed. Hypercholesterolemia (especially high levels of LDL cholesterol), hypertriglyceridemia and diabetes mellitus are major risk factors for cardiovascular disease. In opposition, high levels of HDL cholesterol have been proven to associate to a low risk for cardiovascular disease. Our results demonstrated that hypercholesterolemia patients present a poor periodontal status. We demonstrated in this study a possible role of hyperlipidaemia in the periodontal disease. The pro-atherogenic modifications of plasmatic lipids and blood glucose observed in periodontal disease patients may offer further evidence of a tight association between the periodontal disease and cardiovascular disease. The existence of a relationship between insulin resistance (characteristic of obesity, diabetes and metabolic syndrome) and periodontal disease appears to be central (Minty et al., 2019).

This research direction has been materialized by publishing the following paper:
Martu S, Solomon S, Sufaru I, Scutariu M, Rezus C, Popescu E. The evaluation of the C reactive protein levels in the context of the periodontal pathogens presence in cardiovascular risk patients. Rev. Chim. (Bucharest) 2017; 68(5): 1081-1084.
http://bch.ro/pdfRC/41%20MARTU%20S%205%2017.pdf

I.6.1.1 The evaluation of the C reactive protein levels in the context of the periodontal pathogens presence in cardiovascular risk patients

Aim of the study

We conducted a study with the purpose to investigate the serum C-reactive protein (CRP) values in the presence of *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola* or *T. forsythia* bacteria, as an indicator of the cardiovascular risk in a group of patients aged 55-75 years.

Materials and method

The study group included 64 male and female subjects aged 55 to 75 years. Subjects were periodontally examined, with the determination of the probing depth, the periodontal attachment level, the number of present teeth (except the wisdom molars), with the diagnosis of periodontal disease. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

Subgingival bacterial plaque sampling was performed from sites with the highest depth found in the patient. The sites of interest were isolated with cotton rolls and gently dried with the air spray.

The bacterial plaque was harvested using a single Gracey curette (Hu-Friedy, Chicago, IL, USA) from the base of the pocket to the coronary side. The samples were then placed in phosphate solution and immediately transferred for storage at -80^{0}C until analysis. *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia* and *T. denticola* were detected by real-time quantitative polymerase chain reaction (qPCR).

Subjects were classified as having each pathogen present or not; this was an aggregate of all plaque samples, which means that if at least one sample for a subject was positive, the subject was reported to be positive for the pathogen.

For determination of C-reactive protein, venous blood samples were harvested and centrifuged. Small quantities were then frozen and stored at -80^{0}C until analysis. CRP was measured using Quantex Biokit Reagents.

Body Mass Index (BMI) was calculated as weight / height^2 (kg / m^2). Smokers have been classified as current smokers or not. Diabetes and hypertension were determined by specific measurements (blood glucose and blood pressure, correlated with patient history). A history of cardiovascular disease was recorded for subjects who had a previous myocardial infarction or an intervention such as angioplasty or bypass with stenting of the coronary arteries.

Cerebral-vascular disease was recorded for subjects who had previously had a stroke. Material conditions were classified according to the type of home and lifestyle (rented or owned / mortgaged), the number of cars / vans / motorcycles in the household and the number of bathrooms and / or showers and toilets in the house.

Results

Based on the statistical analysis, there were no significant differences in age, BMI, smoker status or CRP. The mean age of the 64 subjects was 72.5 years, with a range of 55-75 years. Mean BMI was 27.3 kg / m^2, with 21% of subjects classified as obese (BMI ≥ 30 kg / m^2). 21 subjects (30.40%) had mild periodontitis, 32 subjects (50.00%) had moderate periodontitis, and the remaining 11 subjects (19.60%) had severe periodontitis.

These and other characteristics of the subjects studied are presented in Table I.10. The prevalence rates of the pathogens were: 45.0% *P. gingivalis*; 20.5% *A. actinomycetemcomitans*; 86.1% *T. forsythia*; 86.3% *T. denticola*. The median CRP was 1.5 (IQR 1.0-2.6) mg / l.

We analysed the risk levels according to CRP. Following this analysis, 11 subjects (19.6%) had a low risk (<1.0 mg / l); 15 (23.4%) have a medium risk (1.0-3.0 mg / l); and 38 subjects (57.0%) were in a high risk category (> 3.0 mg / l). There was a significant difference in CRP values between subjects who had *P. gingivalis* compared to those who did not (p = 0.003). There were no significant differences for any of the other pathogens (Table I.11).

There was a significant association (p <0.001) for each of the four pathogens investigated with moderate periodontitis. The differences were also significant for severe periodontitis: *P. gingivalis* (p = 0.01), *T. forsythia* (p <0.01) and *T. denticola* (p <0.01), all associated with periodontitis, but not for *A. actinomycetemcomitans* (p = 0.12), as shown in Table I.12.

Table I.10. CRP values based on the presence or absence of the investigated pathogens

	Present pathogen		Absent pathogen		p-Value
	n	CRP (mg/l) Mean	n	CRP (mg/l) Mean	
P.gingivalis	30	2,03 (1,2-3,08)	34	1,53 (0,88-2,32)	0,003*
A.actinomycetemcomitans	12	1,87 (1,04-2,84)	52	1,70 (1,02-2,60)	0,82
T.forsythia	54	1,88 (1,15-2,64)	10	1,87 (0,86-3,60)	0,91
T.denticola	56	1,89 (1,14-2,76)	8	1,80 (1,05-2,61)	0,59
***Statistical significance (p<0,05)**					

Multiple regression analysis showed that the body mass index (p <0.001), current smoking (p <0.01), hypertension (p = 0.01) and the presence of *P. gingivalis* (p <0.01) are independent CRP predictors.

The presence of *P. gingivalis* was associated with a 1.20-fold increase in CRP (95%, confidence interval 1.04-1.37) in the fully-adjusted model. There were no significant associations between the presence of other periodontal pathogens investigated and CRP.

The main finding of this study was that the presence of *P. gingivalis* in the subgingival plate was significantly associated with the C-reactive protein level in a homogeneous group of 55-75 year-olds. This relationship remained significant after adjusting for various bias factors. There were no associations between the presence of several other periodontal pathogens and the level of CRP.

Table I.11. Prevalence of moderate / severe periodontitis depending on the detected pathogen

	Periodontitis prevalence		Odds ration	(95% IC)		p-Value
	Present pathogen	Absent pathogen				
Moderate periodontitis						
P.gingivalis	11	7	2,52	1,72	3,70	<0,001
A.actinomycetemcomitans	5	13	2,10	1,33	3,30	<0,001
T.forsythia	17	2	1,63	1,63	6,24	<0,001
T.denticola	18	1	2,73	2,73	14,03	<0,001
Severe periodontitis						
P.gingivalis	5	4	1,75	1,17	2,82	0,01
A.actinomycetemcomitans	2	6	1,48	0,84	2,56	0,12
T.forsythia	8	1	4,17	1,37	11,65	<0,01
T.denticola	9	1	5,63	1,62	17,22	<0,01

Table I.12. Group characteristics depending on the presence of *P.gingivalis*

	P.gingivalis present	*P.gingivalis* absent	p-Value
Age (yrs) (mean)	73,8	73,2	0,02*
Present teeth (mean)	18,1	19,00	0,08
BMI (kg/m^2) (mean)	26,3	26,1	0,52
Cholesterol (mmol/l) (mean)	5,7	5,7	0,98
Diabetes mellitus (n)	3	2	0,58
Arterial hypertension (n)	7	2	0,89
Smokers (n)	5	2	0,03*
***Statistical significance (p<0,05)**			

Discussion

The bacterial species identified in the "red complex", together with *A. actinomycetemcomitans*, are frequently isolated together and have been strongly associated with periodontal disease. Our hypothesis was that these periodontal pathogens could also be associated with systemic inflammation, measured by CRP. The study confirmed this hypothesis. It was noted that although all four parodontopathic agents were associated with moderate and severe periodontitis (except *A. actinomycetemcomitans*), only *P. gingivalis* was associated with CRP levels. A pattern of pathogenesis of periodontitis has been described - "The key pathogen hypothesis" - suggests that the abundance of microbial pathogens such as *P. gingivalis* can orchestrate periodontal inflammatory disease by remodelling the symbiotic microbiota, normally benign to a dysbiotic form (Curtis et al., 2020).

Data from the Human Microbiome Project (Human Microbiome Project Consortium, 2012) suggests that there is significant diversity in the microfilm of both healthy and diseased periodontium. Much of the research so far supports *P. gingivalis* as a key pathogen. The murine initial model showed that *P. gingivalis* could trigger changes in the amount and composition of oral microbiota, leading to periodontal bone inflammatory changes (Hajishengallis et al., 2011). The main identified pathogenic way was the subvertment of the complement, which led to the creation of a dysbiotic microbiota with the clinical signs associated with the disease. This has also been demonstrated in rabbits (Hasturk et al., 2007), where *P. gingivalis* caused the transition to an anaerobic microbiota and a global increase in bacterial load. Dysbiotic bacterial load may be of greater importance when analysing a systemic inflammatory response than the presence of specific pathogens.

In this study, the hypothesis was whether the current exposure to a pathogen in a cross-sectional model is associated with a high level of systemic inflammation. Exposure was defined by the detectable presence of one of the four pathogens. A possible additional explanation for the association between *P. gingivalis* and elevated CRP values may be found in genetic variants in subjects, which may predispose the development of a dysbiotic microbiota and increase the production of pro-inflammatory cytokines. Nibali et al. (2007) showed that genetic polymorphisms of interleukin-6 were associated with increased detection rates of *A. actinomycetemcomitans*, *P. gingivalis* and *Tannerella forsythia*, after adjusting for age, ethnicity, smoking and the severity of periodontitis. Periodontal disease may, in fact, be just one of the few co-morbidities that develop on the basis of the interaction between microbial dysbiosis and other established risk factors, such as smoking. A good knowledge of the genetic basis of the interaction between the host and the microbe will be necessary to fully understand such mechanisms.

CRP was the main marker investigated in this study, being recognized as an important biological marker of inflammation. Elevated levels of CRP have consistently been shown to increase the risk of cardiovascular disease (Diederichsen et al., 2018) and of type 2 diabetes (Bosevski et al., 2017). Understanding the multidirectional and dynamic links between obesity, metabolic syndrome and periodontal disease can improve the current preventive and therapeutic modalities of these conditions. For example, TNF-α levels of crevicular fluid could be screened in obese individuals to identify a subgroup of obese subjects who are more likely to develop periodontal disease.

Conclusions

Fat tissue produces a large number of cytokines and hormones involved in the inflammatory process. The three conditions are based on the same pathophysiological mechanism. The common factor remains the inflammatory reaction. Relations are bidirectional, it is necessary to understand if one induces the other indirectly or if concomitant they worsen. Of the four periodontal pathogens investigated, only the presence of *P. gingivalis* in subgingival bacterial plaque samples was significantly associated with a high level of C-reactive protein. This relationship remained significant after adjusting for different bias factors. Knowledge and understanding of the relationship between oral microbiota and both periodontal and systemic health will need to be further developed to fully elucidate the mechanisms of potential associations.

I.6.2 The relationship between periodontal disease and chronic kidney diseases

State of the art in the periodontal disease-CKD relationship

Chronic kidney disease (CKD) is defined, regardless of its cause, by the presence for more than 3 months of markers of renal damage or of a decrease in glomerular filtration rate

estimated below 60ml / min / 1.73m^2. These disorders attack the renal filtration, the nephrons and deteriorate the capacity to eliminate waste, and the excess of liquid. Cardiovascular disease (including hypertension) and diabetes are the most common causes, accounting for almost one in two of the stages of end-stage chronic kidney disease (CKD) alone. Chronic renal insufficiency (CRI) is an irreversible alteration of the glomerular filtration system.

The general inflammatory state in CKD is high. CKD and PM are significantly and consistently associated. We are well aware of the effects of CKD on oral tissues: xerostomia, calcification by obliteration of the pulp chamber and root canals, modification of salivary pH. The periodontal prevalence in patients with CKD is high. In addition, periodontitis is an independent risk factor for CKD (Sanz et al., 2020). Its role is difficult to define due to confounding factors such as diabetes or tobacco, but their relationship is indeed two-way (Hajishengallis, 2015).

In order to assess the local periodontal status in haemodialysis patients and to correlate the findings with the quality and quantity of oral hygiene and smoking status, we conducted a study in the Speciality Ambulatory of the „Saint Andrew" Emergency Hospital, Nephrology Section, in collaboration with the Periodontology Clinic of „Grigore T. Popa" UMPh Iași on thirty-six patients with chronic periodontitis and chronic kidney disease. The periodontal status in chronic kidney disease patient who undergo haemodialysis presents severe changes, manifested by a high degree of periodontal tissue loss, accompanied by increased values of plaque and calculus indexes. These data correlate to a poor oral hygiene control and also to heavy smoking status. Also, a high number of patients accused the sensation of xerostomia (dry mouth).

The pathophysiology of the inflammatory response is characterized by:

- Low IL-6 urinary excretion and increased serum levels of pro-inflammatory cytokines
- Oxidative stress, accumulation of AGE caused by a decrease in renal clearance
- And the presence of comorbid factors (diabetes, atherosclerosis, etc.).

We know that periodontal disease has an increase in IL-6 and CRP that causes an inflammatory reaction on a systemic level. The periodontitis/CKD relationship increases CRP levels synergistically and promotes a general inflammatory state. By promoting systemic inflammation, periodontal disease can worsen CKD.

In a study conducted on 69 patients with terminal CKD, who were following hemodialysis regime in the Fresenius Hemodialysis Center of "Doctor C.I. Parhon" Clinical Hospital, Iasi, we observed that the mean value of the CRP levels was of 0.608mg/dl. The measured values revealed relatively normal intervals (85.7% presented values under 1.00 mg/dl) (Solomon et al., 2015).

The infectious response of CKD increases as we have seen the concentration of pro-inflammatory cytokines but also the bacterial load. If oral hygiene is unsatisfactory, there may be a greater development of periodontal disease (Chambrone et al., 2013).

According to the study by Ioannidou and Swede (2011), a dose-response relationship between periodontal disease and the different stages of CKD was observed, and they found that people with CKD were 30 to 60% more likely to develop periodontitis. A few years later, it has been shown that the Mexican population with impaired renal function is twice as likely to have periodontal disease as subjects with normal renal function (Ioannidou et al., 2013). In a 2015 prospective cohort study with 14 years of follow-up, the authors found that subjects with CKD and periodontitis had a 35% higher risk of mortality than patients with disease-free CKD periodontal (Ricardo et al., 2015).

Periodontal treatment has a positive effect on the glomerular filtration rate, but it is necessary to ensure regular monitoring. Indeed, the bacterial load after 3 months of treatment in a patient with CKD is likely to return to the subgingival fluid (Chambrone et al. 2013).

This research direction has been materialized by publishing the following papers:

1.Veisa G, Donciu MD, Segall L, Hurjui L, Nistor I, Ursarescu IG, Solomon SM. Albumin as a prognostic factor for malnutrition and inflammation in chronic kidney disease. Rev. Chim (Bucharest) 2016; 67(1):103-105.
http://www.revistadechimie.ro/pdf/VEISA%201%2016.pdf
2.Veisa G, Tasmoc A, Nistor I, Segall L, Siriopol D, Solomon SM, Donciu MD, Voroneanu L, Nastasa A, Covic A. The impact of periodontal disease on physical and psychological domains in long-term hemodialysis patients: a cross-sectional study. Int Urol Nephrol 2017; 49: 1261-1266.
https://link.springer.com/content/pdf/10.1007/s11255-017-1571-5.pdf
3.Solomon SM, Sincar C, Rudnic I, Pasarin L, Ursarescu IG, Martu MA, Ioanid N, Martu S. The assessment of renal biochemical markers and their role in periodontal disease. Rom J Oral Rehab 2015; 7(3): 97-102.
http://www.rjor.ro/the-assessment-of-renal-biochemical-markers-and-their-role-in-periodontal-disease/?lang=ro
4.Solomon S, Forna N, Ursarescu I, Segal L, Nistor I, Veisa G. The oral cavity status in patients with end stage kidney disease and hemodialysis, in corelation to the history of renal impairment and C-reactive protein levels (Pilot study). Rom J Oral Rehab 2014; 6(1): 9-14.
http://www.rjor.ro/the-oral-cavity-status-in-patients-with-end-stage-kidney-disease-and-hemodialysis-in-correlation-to-the-history-of-renal-impairment-and-c-reactive-protein-levels-pilot-study/?lang=ro

I.6.2.1 Albumin as a prognostic factor for malnutrition and inflammation in chronic kidney disease

Aim of the study

Our objective was to evaluate for the first time, the relationship between albumin level, malnutrition and periodontal status in a Romanian cohort of haemodialysis patients.

Materials and method

We conducted a prospective study, that comprised hemodynamic stable patients on haemodialysis treatment for at least six months, recruited from two dialysis units auxiliary to ‘’Dr. C. I. Parhon’’ University Hospital, Iasi, Romania. The patients` observation period was between March 2013 to October 2015. Participants gave informed consent before enrolling in the study. The study was approved by the Hospital’s Ethical Committee and was performed in agreement with Helsinki’s declaration of human rights. The exclusion criteria were age under 18 years, ongoing acute illnesses, and who declined to participate in the study. All patients received HD a four hours/session, three times/week. Laboratory measurements for biochemical data were taken under fasting pre-dialysis conditions, but not necessarily on a midweek dialysis day.

The analysis was made with SPSS 20.0 statistical standard package for Mac OS X and a p value < 0.05 was considered statistically significant. Descriptive statistics of the demographic data of the study population and Pearson's` correlation were calculated. Baseline characteristics of the study sample, assessed by descriptive statistics, are presented as means ± standard deviation (SD) or as percent, as appropriate. Body weight (BW) was measured at the end of a HD session (i.e., the ‘‘dry weight’’). In the end, patients were classified into three categories (‘‘the SGA mark’’), as follows: A (well nourished), B (mild PEW), and C (severe PEW). Also, serum albumin level was measured. Regarding the serum markers of inflammation in haemodialysis patients, these included C-reactive protein,

interleukin 6 and TNF α. The data were recorded in individual observation charts and statistically analysed.

Results

This prospective study included a total of 200 patients (101 males) (the general characteristics of the study sample are summarized in Table I.13).

Table I.13. Baseline general characteristics of the study sample (mean ± SD and N, % as appropriate)

General baseline characteristics	Total (N=200)
Males (n, %)	101 (50.5)
Age (years)	54.11 ± 14.37
Living environment (urban, n, %)	91 (45.5)
Vascular access (arterio-venous fistula, n, %)	187 (93.5)
Smoking status (n, %)	36 (18)
Dialysis vintage (years)	5.6 ± 5.2
Kt/v	1.38 ± 0.24
Serum Hb (g/dl)	11.65 ± 1.65
Serum Ferritin (ng/ml)	36.43 ± 8.26
TSAT (%)	28.68 ± 15.53
Serum total cholesterol (mg/dl)	182.54 ± 45.0b
Predialysis HCO3 (mmol/l)	22.63 ± 2.9
Serum Phosphate (mg/dl)	5.25 ± 1.48
Serum iPTH (pg/ml)	828.81 ± 712.36

The mean age was 54.11 ± 14.37 years, mean Kt/v 1.38 ±0.24 and mean Hb g/dl was 11.65±1.65. Patients had received renal replacement treatment (HD) for a mean duration of 5.6 ± 5.2 years. According to inflammatory markers the means values were: CRP (mg/l) 9.55 ± 14.80, IL-6(pg/ml) 285.76 ± 433.98, TNF α (pg/ml) 481.127 ± 839.70. In terms of nutritional markers, mean Albumin (g/dl) was 4.24±0.43 and mean of total score of SGA "7-point scale" was 2.5 ±1.63 (Table I.14).

Table I.14. Characteristics and scores of inflammatory markers, nutritional markers and periodontal evaluation in the study sample (mean ± SD)

Inflammatory markers	**Total (N=200)**
CRP (mg/l)	9.55 ± 14.80
IL-6 (pg/ml)	285. 76 ± 433.98
TNF α (pg/ml)	481.127 ± 839.70
Nutritional markers	**Total (N=200)**
Albumin (g/dl)	4.24 ± 0.43
SGA (''7 points scale'' - total score)	2.5 ± 1.63
Periodontal evaluation	**Total (N=200)**

Table I.15 shows the correlation coefficients between inflammation markers (protein C-reactive (mg/l), Il-6(pg/l), TNF- α(pg/ml) and relevant variable (TSAT %, SGA, dialysis vintage). First inflammation marker, CRP, was positively correlated with Il-6 (r= 0,53, p<0.01), SGA (r=0.02, p<0.01), TNF-α (r=0.57, p<0.01); second, Il-6, positively correlated

with dialysis vintage (r=0.22, p<0.01), TNF-α (r=0.49, p<0.01), SGA (r=0.21, p<0.01); and the last one, TNF-α, positively correlated with SGA (r=0.17, p<0.05).

Table I.15. Association of each inflammation markers with nutritional markers

Total (N = 200)	Dialysis vintage (years)		TSAT (%)		Albumin (g/dl)		CRP (mg/l)		IL-6 (pg/ml)		TNF α (pg/ml)		SGA (total score)		Hospitalizations for respiratory infections		Hospitalizations for urinary infections		Hospitalizations for CV impairment	
Inflammation markers	Pearson's r coefficient, p value																			
CRP (mg/dl)	0.04	0.5	-0.15*	<0.05	-0.23*	<0.05	-	-	0.53**	<0.01	0.57**	<0.01	0.20**	<0.01	0.35**	<0.01	0.08	0.2	0.28**	<0.01
IL-6 (pg/ml)	0.22**	<0.01	-0.22**	<0.01	-0.94	0.18	0.53**	<0.01	-	-	0.49*	<0.01	0.21**	<0.01	0.12	0.09	0.12	0.08	0.13	0.05
TNF α (pg/ml)	0.04	0.53	-0.18*	<0.05	-0.06	0.3	-0.57**	<0.01	0.49**	<0.01	-	-	0.17*	<0.05	0.17*	<0.05	0.14*	<0.05	0.18**	<0.01

Table I.16 shows the correlation coefficients between nutritional markers (Albumin g/dl), SGA (total score), BMI (kg/m²) and relevant variable (age, CRP, dialysis vintage, TSAT, GPC, BPC).

The nutritional marker, SGA, was positively correlated with age (r=0.14, p<0.01) and with dialysis vintage (r=0.19, p<0.01). Another nutritional marker, BMI, was positively correlated with age (r=0.28, p<0.01), with GPC (r=0.16, p<0.05) and with BPC (r=0.14, p<0.05).

Table I.16. Association of each nutritional markers with clinical and biochemical parameters, and periodontal evaluation

Total (N = 200)	Age (years)		Dialysis vintage (years)		CRP (mg/l)		IL-6 (pg/ml)		TNF α (pg/ml)		TSAT (%)		CGP		CPB		Hospitalizations for respiratory infections		Hospitalizations for CV impairment	
Nutritional markers	Pearson's r coefficient, p value																			
Albumin (g/dl)	-0.30**	<0.01	-0.05	0.4	-0.23**	<0.01	-0.09	0.18	-0.06	0.39	-0.05	0.41	-0.05	0.44	-0.13	0.06	-0.07	0.28	0.17*	<0.05
SGA (total score)	0.14**	<0.05	0.19**	<0.01	0.20**	<0.01	0.21**	<0.01	0.17*	<0.05	-0.12	0.07	-0.02	0.68	-0.04	0.55	0.20**	<0.01	0.29**	<0.01
BMI (kg/m²)	0.28**	<0.01	-0.09	0.19	0.11	0.11	-0.006	0.93	-0.09	0.17	-0.16*	<0.05	0.16*	<0.05	0.14*	<0.05	0.00	0.98	-0.006	0.93

During study follow-up (two years) we registered 14 deaths in total, most of them (ten out of 14) due to cardiovascular diseases. Only for two patients the cause of death was infection.

Discussion

This pilot study prospectively examined in a cohort of 200 HD patients, for the first time, the relationship between baseline nutrition and inflammatory markers in a population with chronic kidney disease.

We found that inflammation (CRP, IL-6, TNFα) is linked with anaemia (decreased of TSAT) or malnutrition (SGA increase). When looking at nutritional status, we found that malnutrition is linked with age (albumin, SGA, BMI) and CRP (SGA, albumin).

Previous studies found that CKD patients have high level of inflammation and this might be linked with mortality (Bernelot Moens et al., 2017). In our analysis, SGA increase with one unit is associated with 62% higher mortality. Also, albumin increase with 1g/dL is associated with 79% lower mortality.

Our results are similar with the recent work of Pereira and collaborators (Pereira et al., 2015). They showed that the prevalence of sarcopenia in CKD patients on conservative therapy varies according to the method applied.

Sarcopenia defined as reduced handgrip strength and low skeletal muscle mass index estimated by BIA was an independent predictor of mortality in these patients.

The strength of our study included the multidisciplinary approach and global evaluation of nutrition and inflammation with old and new markers (SGA, albumin, TNF-α, IL6, CRP, bioimpedance). The second strength is linked with the systematic approach and evaluation of the periodontal disease.

The limitations of our study are related with the observational study design. All our findings must be interpreted with caution since other co-factors might explain the results.

Conclusions

In our HD patients, albumin and nutritional status (evaluated by SGA score) were associated with a significantly increased death risk. Further evidence is needed in order to support inflammation markers as a long-term predictor for decline in ESRD patients.

I.6.2.2 The evaluation of the interrelation between chronic periodontitis and chronic renal disease by quantifying the renal biochemical markers

Aim of the study

The aim of the study was to assess the interrelation between the periodontal disease and the chronic kidney disease by an evaluation of renal biochemical markers. Another objective of this study was to evaluate the prevalence and severity of periodontal disease and its relationship with the quality of life (QoL) in a cohort of haemodialysis patients.

Materials and method

The study was conducted in the Speciality Ambulatory of the „Saint Andrew" Emergency Hospital, Nephrology Section, in collaboration with the Periodontology Clinic of „Gr.T.Popa" UMPh Iași.

All the patients had the ages between 32 and 58 years old.

- Test group: Thirty-six patients with chronic periodontitis and chronic kidney disease were selected for the study group.
- Control group: Thirty systemically healthy subjects were included, presenting periodontal disease.

The exclusion criteria were represented by:

- Unfavourable systemic diseases (rheumatic fever or heart conditions which require prophylactic therapy with antibiotics)
- Pregnancy
- Women with hormonal substitution therapy or oral contraceptives
- Patients with steroidal or non-steroidal anti-inflammatory drug therapy (in the last 3 months) or antibiotics (in the last 6 months)
- Smoking and
- Patients with high sensitivity on C-reactive protein (CRP), higher than 10mg/l, conditions which can affect the evolution of periodontitis.

In order to assess the degree of metabolic control and to establish the evolution stage of

CKD and of periodontal disease, a rigorous clinical examination was conducted, completed by laboratory investigations and speciality inter-clinical consultations. A research sheet was filled in for each patient.

The data regarding the history and evolution of CKD were obtained from the anamnesis and the nephrology clinical hospital sheet of the patient.

The study methodology respected the Helsinki Declaration norms. Before any type of investigation, the informed consent was obtained from the patients/parents/tutors and also from the speciality doctors who took care of the hospitalized patients.

For the periodontal recording all 4 surfaces of the tooth were examined and the oral cavity was divided in 4 quadrants.

The periodontal disease indexes were the following:

- Plaque index (PI)
- Papillar bleeding index (PBI)
- The clinical attachment loss, measured by periodontal probing and radiologic examination (AL)
- Periodontal pocket depth (PPD)

Venous blood samples (8ml in tube) were taken from patients, after a 12 hours period of fasting during night-time. The 24 hours collecting time for urine was completed in the morning of the blood collecting. The blood and urine samples were analyzed immediately after their collection (Figure I.30a).

The serum and urinary urea were determined by measuring the UV absorption of consumed NADH in the presence of glutamate dehydrogenase.

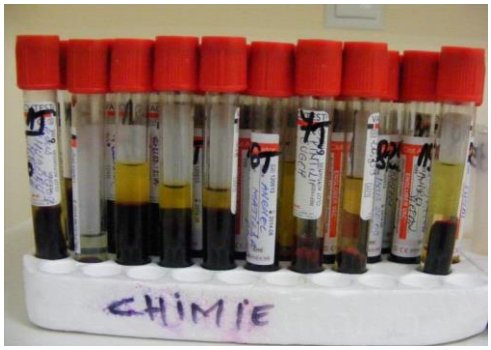
The creatinine was analysed by Lustosa-Basques colorimetric method, based on the absorbance of complex and colorants formed by picrate creatinine with interference compounds on alkaline pH; when the acetic acid is added, the picrate creatinine complex is destroyed and the difference between the two absorbents is proportional with the creatinine quantity.

The uric oxidase test was employed to measure the uric acid. The uric acid is the first one to be oxidised, while the oxygen is transformed in hydrogen peroxide which is determined by the reaction with peroxidise and a chromogenic substance.

The serum albumin is determined by the reaction with green bromcresole, which form a complex with the albumin which absorbs a certain wavelength in visible spectrum. The urine albumin was analysed by a quantitative immune-nephelometric method (N anti-serum to human albumin), following the producer instructions (Dade Behring GmbH, Marburg, Germany).

The serum and urinary levels of renal function markers (urea, creatinine, albumin and uric acid) were measured and the glomerular filtration rate was estimated by eliminating the creatinine from the Modification of diet in renal disease (MDRD) and of the albumin/creatinine fraction in 24h urine sample.

The serum and urinary urea, creatinine and albumin analysis were conducted in a Daytona analyser (Figure I.30b). The serum and uric acid reagents were from Kovalent do Brasil Ltda. (Sao Goncalo, Rio de Janeiro), and those for serum albumin, urinary urea and creatinine were from the Labtest Diagnostics S.A. kit (Belo Horizonte, Brasil).



b

Figure I.30. a.Venous blood samples; b.Daytona analyser

To assess the QOL, we used the Short Form 36-Item Health Survey (SF-36), a generic instrument, translated and validated in Romanian patients with ESKD [22]. This instrument is divided into 8 dimensions: physical functioning, physical role functioning, pain, general health, vitality, social role functioning, emotional role functioning and mental health. The results vary on scales from 0 to 100 (i.e., from worse to best possible status).

The physical and mental components of the 8 dimensions were combined into a physical component summary (PCS) and a mental component summary (MCS), respectively.

We observed that the immunologic and the inflammatory values were comparable for both study groups. The mean values were beyond the upper physiologic limit.

Results

The gender distribution was similar for the two groups: 13 male and 17 female subject in the control group and 20 male and 6 female subjects in the study group. There was no significant difference between the age mean and standard deviation (SD) values for the groups: 43 ±5 years and 46 ±6 years, respectively.

The subjects presented a mean PI value of 58.00±20.7% and 63.5±16,4% bleeding areas; the mean periodontal pockets number was of 77±23, the clinical attachment loss of 4,93±1,13 mm and periodontal probing depth of de 4,36±0,59mm (Table I.17).

Table I.17. Periodontal parameters in the study groups

Parameter	Test group (mean± standard deviation)	Control group (mean± standard deviation)
No. of periodontal pockets >4mm	77.08±23.23	27.82±16.36
Pockets depth (mm)	4.36±0.59	3.25±0.47
Attachment loss (mm)	4.93±1.13	4.74±1.14
Recession (mm)	0.56±0.88	1.56±0.94
Plaque index (%)	58.04±20.70	20.90±14.29
Bleeding index (%)	63.57±16.39	15.80±11.57
Calculus index	61.30±23.40	54.60±8.70

The PPD values were classified as follows: <3mm (superficial), 3-5mm (moderate) and ≥6mm (deep); the classes presented a high variance between groups and only the test group presented deep periodontal pockets.

The number of teeth and the proportion of superficial periodontal pockets were higher for the control group, while higher values for the other variables were encountered in the test group (p=0.006).

Serum and urinary levels of the markers of the renal function are described in Table I.18.

Regarding the metabolic control among the study population, we observed slightly more favourable values for the biochemical markers for the systemic healthy subjects,

indicating a satisfactory metabolic control. A slightly significant difference was present between groups for the mean values of all the biomarkers. This aspect was present also for the glomerular filtration rate (Table I.19).

Table I.18. Serum and urinary markers for the study groups

Marker	Test group	Control group
Serum albumin (g/dl)	4.7±0.4	4.9±0.5
Serum creatinine (g/dl)	1.0±0.2	0.9±0.2
Urinary creatinine (mg/kg.24h)	17.14±4.75	18.45±4.30
Serum uric acid (mg/dl)	4.9±2.1	4.3±1.7
Urinary uric acid (mg/24h)	526.5±222.1	542.2±219.0
Serum urea (mg/dl)	29.00±10.00	30.00±9.00
Urinary urea (mg/24h)	24.5±11.0	25.9±8.00

Table I.19. Glomerular filtration rate estimated in control and test subjects

Variable	Test group	Control group
Creatinine Clearance (ml/min/1.73m^2)	85.28±21,74	91,37±22.05
MDRD2 (ml/min/1.73m^2)	78.16±12,03	81.27±13.28
Urinary Albumin / Creatinine (mg/g)	5.53±4.72	4.87±3.25
MDRD2: Modification of diet in renal disease study. **The values are expressed as mean ± standard deviation.**		

Table I.20. The frequency and the percentage of patients in control and periodontitis patients for whom the biochemical marker values are presented inside and in their reference intervals of normal values

Variable	Class	Test		Control		RI
		Fr	%	Fr	%	
Serum albumin (g/dl)	Inside RI <RI >RI	25 0 5	83.3 0.0 16.7	21 1 8	70.0 3.3 26.7	3.5-5.2
Serum creatinine (mg/dl)	Inside RI <RI	29 1	96.7 3.3	31 2	98.4 3.1	M: 0.9-1.3 F:0.6-1.1
Urinary creatinine (mg/kg.24h)	Inside RI <RI >RI	9 20 1	30.0 66.7 3.3	11 16 3	36.7 53.3 10.0	M: 21-26 F:16-22
Serum uric acid (mg/dl)	Inside RI <RI >RI	21 5 4	70.0 16.7 13.3	23 4 3	76.7 13.3 10.0	M: 3.5-7.2 F:2.6-6.0
Urinary uric acid (mg/24h)	Inside RI <RI	29 1	96.7 3.3	28 2	9.3 6.7	<1000
Serum urea (mg/dl)	Inside RI <RI >RI	25 0 5	83.3 0 16.7	25 1 4	83.3 3.3 13.3	15-40
Urinary urea (mg/24h)	Inside RI <RI >RI	8 18 4	26.7 60.0 13.3	12 17 1	40.0 56.7 3.3	26-43

RI: reference interval. Fr: frequency (number of patients from each class regarding the RI). M: male. F: female. Statistic analysis: c2 test (P<0.5)

The absolute frequencies and the percentages of the indicators of the renal function inside and outside the reference intervals for the two groups are presented in Tables I.20 and I.21.

Table I.21. The frequency and the percentage of patients in control and periodontits patients for whom the biochemical marker values are presented inside and in their reference intervals of normal values

Variable	Class	Test		Control		RI
		Fr	%	Fr	%	
Creatinine Clearance (ml/min/1.73m^2)	Inside RI	20	66.7	23	76.7	35–44 ani: 74 – 138
	<RI	9	30.0	5	16.7	45–54 ani: 74 – 129
	>RI	1	3.3	2	6.7	55–64 ani: 69 – 122
MDRD2 (ml/min/1.73m^2)	Inside RI	20	66.7	20	66.7	35–44 ani: 74 – 138
	<RI	10	33.3	10	33.3	45–54 ani: 74 – 129
						55–64 ani: 69 – 122
Urinary Albumin / Creatinine (mg/g)	Inside RI	30	100.0	30	100.0	≤ 30

MDRD2: Modification of diet in renal disease study. RI: reference interval. Fr: frequency (number of patients from each class regarding the RI). Statistic analysis: c2 test (P<0.5)

No matter the analysed variable, there is no proof that the frequency of the results inside and outside the reference interval is different between the groups. The same was observed for the glomerular filtration rate (Table I.22).

Table I.22. Inflammatory markers values on study groups

Marker	Study group	Control group	p-value
C reactive protein (mg/l)	5.02±6,0	1.96±2.1	<0.01
ESR (mm/h)	7.4±10.6	6.3±9.7	<0.001
Fibrinogen (mg/dl)	309.7±94.1	264.0±73.7	<0.05

ESR: erythrocyte sedimentation rate
The values are expressed as mean ± standard deviation.

Table I.23. Mean and standard deviation of QoL and periodontal parameters in hemodialysis patients (N = 101)

Quality of life components	
Physical component summary (PCS) score	38.00 ± 17.29
Mental component summary (MCS) score	45.04 ± 16.30
Periodontal evaluation	
GP	4.02 ± 1.28
PI	1.76 ± 0.90
CI	1.33 ± 0.72

GP gingival and periodontal index, PI bacterial plaque index, CI calculus index

Table I.24. Baseline characteristics of clinical parameters and QoL components scores in subgroup without periodontitis and group with periodontitis

	Group 1 (N = 25) GP rank 0–3	Group 2 (N = 76) GP rank 3–6	p
Age	42.16 ± 17.13	55.83 ± 11.51	<0.001
Mg	2.92 ± 0.48	2.66 ± 043	0.014
Mental component summary (MCS) score	56.36 ± 18.65	41.80 ± 13.77	<0.001
Physical component summary (PCS) score	8.28 ± 19.54	34.61 ± 15.15	<0.001

Mg magnesium, GP gingival and periodontal index, rank 0–3 absence of periodontitis, rank 3–6 presence of periodontitis

We identified 76 patients (75.2%) with GP scores higher than 3.00, suggesting the presence of periodontitis. According to periodontal status, the mean value of GP was 4.0 ±

1.3, mean PI was 1.8 ± 0.9, and mean CI was 1.3 ± 0.7. Xerostomia was experienced by 52.5% of patients. 13.3% of patients reported that they never underwent a dental consultation before, while 83.3% were seen by a dentist only in case of emergency (Table I.23).

Analysing the two components of the QoL questionnaire, the means for PCS and MCS were 38.0 ± 17.3 and 45.0 ± 16.3, respectively. The mean scores and standard deviation of the two domains of the SF-36 and of the three components of periodontal disease are shown in Table I.24

We performed a comparison between patients with no periodontitis (GP score 0–3) and with periodontitis (GP score 3–6). Patients with higher GP scores were older, had a low level of Mg and had low scores at both QoL components (Table I.24).

Table I.24. Univariate associates of physical component of QoL in hemodialysis patients (N = 101)

Variables	Coefficient	p
Age (years)	-0.49	<0.001
GP	−0.27	0.006
PI	-0.21	0.034
CI	-0.25	0.01
Log GP	-0.34	0.001
Log CI	-0.24	0.02
BMI (kg/m^2)	-0.19	0.04
Log CRP	-0.25	0.01
Log magnesium (mg/dL)	0.19	0.04
Atrial fibrillation, 0—no; 1—yes	-0.37	<0.001
Cardiac failure, 0—no; 1—yes	-0.22	0.03
Ischemic cardiomyopathy, 0—no; 1—yes	-0.32	0.001
Diabetes, 0—no; 1—yes	-0.22	0.03
GP gingival and periodontal index, PI bacterial plaque index, CI calculus index, BMI body mass index, CRP C-reactive protein		

Table I.25. Univariate associates of mental component of QoL in haemodialysis patients (N = 101)

Variables	Coefficient	p
Age (years)	-0.37	<0.001
GP	-0.37	<0.001
PI	-0.21	0.03
CI	-0.25	0.01
Log GP	-0.45	<0.001
Log CI	-0.24	0.02
Fat tissue (kg)	0.22	0.03
BMI (kg/m^2)	-0.22	0.03
Angina pectoris, 0—no; 1—yes	-0.21	0.04
GP gingival and periodontal index, PI bacterial plaque index, CI calculus index, BMI body mass index		

Table I.26. Determinants of mental component of QoL in hemodialysis patients (N = 101)−R2 = 0.25

Variables	Backward stepwise multiple regression		
	Coefficient	95% CI	p
Age (years)	-0.28	−0.50 to −0.06	0.01
Log GP, per 1 SD increase	-5.57	−8.74 to −2.41	0.001
GP gingival and periodontal index			

Univariate analysis showed a significant association between the two QoL components with the following parameters: C-reactive protein, magnesium, atrial fibrillation, cardiac failure, ischemic cardiomyopathy, angina pectoris, diabetes, fat tissue, body mass index. For the all the other parameters tested in the univariate analysis, the results were not significant

(Tables I.24, I.25).

The physical and mental components of QoL were significantly associated with the gingival and periodontal index, the bacterial plaque index and the calculus index (Tables I.26, I.27).

Table I.27. Determinants of physical component of QoL in hemodialysis patients (N = 101)−R2 = 0.33

Variables	Backward stepwise multiple regression		
	Coefficient	95% CI	p
Age (years)	-0.45	−0.69 to −0.21	<0.001
Log GP, per 1 SD increase	-3.26	−6.39 to −0.13	0.04
Atrial fibrillation, 0—no;1—yes	-10.73	−20.14 to −1.32	0.03
GP gingival and periodontal index			

In the multivariable linear regression, only the gingival and periodontal index remained significantly associated with both physical (Table I.26) and mental (Table I.27) components of QoL.

The C-reactive protein (CRP) had a mean value for the control group which was lower than the value for the study group. The difference for the ESR and fibrinogen values were significantly higher for the study groups; the low CRP value is certainly an effect of the systemic health (p=0.032660). Knowing that CRP is a marker of the disease activity, these results confirm the fact that CKD can influence the immune response mediators.

Discussion

The two study groups were significantly distinct, with clear signs of severe periodontitis for the study group and the lack of those for the control group. Our results support the literature data (Deschamps-Lehhardt et al., 2018).

The study model can significantly influence the results. An association between periodontitis and CKD is frequently encountered in studies on already diagnosed subjects. In these cases, the lasting of the end-stage and the type of systemic treatment can significantly affect the association (Zhang et al., 2017).

Nevertheless, even in the study model with end-stage patients, there are controversies regarding the association because there were end-stage CKD cases without severe periodontal damage (Solomon et al., 2014).

The cardiovascular complications and the infectious diseases are frequent in patients with CKD, being associated with severe clinical effects. The cellular and humoral anomalies and the phagocytic activity associated with the uremic level may explain the high frequency of aggressive periodontal pathogens in these patients.

Numerous studies presented correlations between the poor oral status and CKD (Avesalon et al., 2012), when compared to healthy subjects. Moreover, an association between high levels of C reactive protein and periodontal disease in CKD patients was presented (Kapellas et al., 2019). Fisher et al. (2008) demonstrated that chronic periodontitis is an independent risk factor for CKD.

In a previous study (Martu et al., 2014) we observed that more severe forms of chronic periodontitis were exerted by CKD patients. Also, in the same study, we observed a high level of association between the presence of the periodontal pathogens from the red complex (*P. gingivalis, T. forsythia* and *T. denticola*) and the systemic context of CKD, more significant when compared to the control group which included healthy subjects. It is possible that the periodontal therapy could improve the prognosis in patients with CKD, by reducing the inflammatory loading but this aspect necessitates further studies.

The periodontitis can predict the open development of nephropathy and the end-stage CKD in type 2 diabetes mellitus patients. In groups such as diabetic HD patients the associations become difficult to analyse and discuss.

Having in mind the limitation of our cross-sectional study, the severe periodontitis was correlated on a moderate level to the renal dysfunction in the study population. Further studies assessing the therapy effects could bring important support to our results.

Mean PCS and mean mental MCS in our population were consistent with a low QoL, although we included a younger population (mean age 52.45 ± 14.31), with a mean dialysis vintage of approximately 7 years. Our findings are not singular in suggesting lower QoL in various HD populations (Yusop et al., 2013).

Our study has several strengths and limitations. To our knowledge, there has never been a comprehensive study to assess a link between periodontal disease and quality of life, assessed with the Short Form 36-Item Health Survey. The study limitations include a small sample size and lack of dental radiographic examination for a more accurate periodontal diagnosis. Being a cross-sectional study, no causal relationship could be explored. We need further longitudinal studies to highlight the predictive relationship between PoD and QoL. Another limitation was the fact that we were not able to explore the etiology of PoD.

The results of the present study show a greater prevalence and severity of periodontal disease in a young HD population. Our HD patients presented weak attitudes and negligence toward oral health. Also, we found that the more severe the periodontal disease, the lower the quality of life in our patients. The gingival and periodontal index was considered a strong predictor for low QoL, with impact on both physical and mental components.

Although previous reports have revealed similar results concerning QOL or PoD, no other studies analysed the impact of PoD on health-related quality of life, using an objective tool—the SF-36 questionnaire for HD patients. Future studies should assess the relative impact of QOL and PoD on patients' survival and cardiovascular morbidity.

In our HD patients, albumin and nutritional status (evaluated by SGA score) were associated with a significantly increased death risk. Further evidence is needed in order to support inflammation markers as a long-term predictor for decline in ESRD patients. The results demonstrated that the patients with CKD presented changes in serum and urinary markers of renal dysfunction, compared to the control group, suggesting the fact that periodontitis can change in certain conditions the renal function. In our opinion, the study model used to assess the correlation between periodontitis and renal disease can be influenced by certain factors, such as smoking, pregnancy, antibiotics. Therefore, the correlation between periodontitis and CKD in healthy subjects can lead the way for damaged kidneys to influence the evolution of periodontitis and vice versa. Also, we found that the more severe the periodontal disease, the lower the quality of life in our patients. The gingival and periodontal index was considered a strong predictor for low QoL, with impact on both physical and mental components.

Development and progression of periodontal disease in an individual are "personalized" by a number of endogenous and exogenous factors. Assessment, knowledge and proper management of these factors facilitate the prevention of disease or its containment in the case of an existing periodontal condition. The medical management of CKD depends on the stage of disease and clinical status of the patient. Patients with CKD under haemodialysis require special consideration with regard to the risk of excessive bleeding or infection and drug therapy. The bleeding tendency in these patients is generated by the use of anticoagulants and maintenance of vascular access.

Conclusions

The long-term effective bacterial plaque control is a necessity for the chronic kidney disease patient. An improved understanding of the systemic and oral changes in patients with kidney disease will help dentists and oral healthcare workers to render efficient oral care and plan preventive regimens tailored to individual needs. Knowing that the signs and symptoms

of renal disease can be detected early in the oral cavity, the dentist can play an important part in the diagnosis and treatment of these patients.

I.6.3 The relationship between periodontal disease and osteo-articular diseases

State of the art in the periodontal disease - osteo-articular disease

Osteoporosis

According to the WHO, osteoporosis is a generalized disease of the skeleton, characterized by low bone density and alterations in bone microarchitecture, responsible for exaggerated bone fragility and therefore a high risk of fracture. Biomechanical studies show that bone mineral density (BMD) is the essential determinant of bone fragility. Fractures are the main complication of osteoporotic disease and constitute the entire gravity of this disease. There is a decoupling between bone formation (by osteoblasts) and bone resorption (osteoclasts). There are two types of primary osteoporosis: type I post-menopause in women, and type II in the elderly. There is also secondary osteoporosis due to treatments such as corticosteroid therapy. It is to be distinguished from osteopenia which is a physiological decrease in bone density, a precursor of osteoporosis.

Osteoporosis and periodontal disease share many risk factors: age, genetics, hormonal changes, tobacco, calcium and vitamin D deficiency. The bone cortex represents an important component of the maxillary bones; it is affected by any skeletal bone impairment. While on young ages the formatory, apposition and functional restructuration are the main processes, the involution and bone mass loss are predominant on old ages. Apart from the causal factor of periodontal disease (the bacterial factor), the existence of local and systemic risk factors was clearly demonstrated.

Osteoporosis is a disease with reduced bone density and quality, leading to a skeletal fragility, with a great bone fracture risk (especially of the spine, wrist and hip) (Paramashivaiah et al., 2011). The risk for osteoporotic fractures ranges for women from 30 to 50% and for men from 15 to 30%. Theoretically, with a less initial bone quantity, the same bacterial infection could generate a faster resorption of the alveolar bone. The osteoporosis prevalence grows within the old age patients and the periodontal disease and the osteoporosis present a great incidence (more of 72% of over 35 years old population is suffering from different forms of periodontal disease), therefore we conducted a study on 52 female subjects with the purpose to examine the relation of osteoporosis as a risk factor for periodontal diseases and to understand if its presence is an important issue for the periodontal treatment and prophylaxis.

An important result of our study consists in the strong correlation between the local factors and the periodontal attachment and bone loss. Moreover, the attachment loss was strongly associated with the bone resorption index. These results confirm the fact that the periodontal status is highly influenced by the local factors. We didn’t notice a relationship between the alveolar bone loss and the bone mineral density. Also, a relationship between the bone mineral density and the attachment loss wasn’t observed. The results of the present study come in concordance with those obtained by Paramashivaiah et all in a study conducted on a group of osteoporotic female subjects in 2011 (Paramashivaiah et al., 2011).

Persson et all evaluated in 2002 in a study conducted on a large number of subjects with osteoporosis the relation between the bone mineral density and the alveolar bone loss (Balcikoyte et al., 2004); contrary to the present study, they concluded a strong relation between these two variables but their study was based only on radiographs, without the clinical parameters, limiting their results signification.

The same pro-inflammatory cytokines are involved: IL-1, IL-6, TNF-α, RANK, RANKL, OPG in both pathologies (osteoporosis and periodontitis), which could explain this association.

Estradiol (a steroid hormone derived from cholesterol necessary for the maintenance of fertility and secondary sexual characteristics in women) inhibits the expression of certain cytokines. After menopause, its rate decreases and there is a lifting of inhibition and therefore an increase in the release of certain cytokines at the local level as at the systemic level (Bouchard, 2015).

Oestrogen deficiency would promote periodontitis either by causing the increased expression of proinflammatory cytokines or by reducing the bone mass of the maxilla. In animal models, oestrogen deficiency exacerbates the severity of periodontitis. Ovariectomized rats have a higher expression of IL-6, RANKL, of osteoprotegerin (OPG) in periodontal tissue, suggesting the impact of the hormone oestrogen on inflammatory bone resorption (Wang & McCauley, 2013).

It would also be interesting to know the impact of osteoporosis on tooth loss and whether it is associated with osteoporotic fractures. Age is a major confounding factor (Han et al., 2014). However, there seems to be a significant association according to a 2016 study, there is a relationship between the two bone resorption disorders (Wang & McCauley, 2013) (Figure I.31).

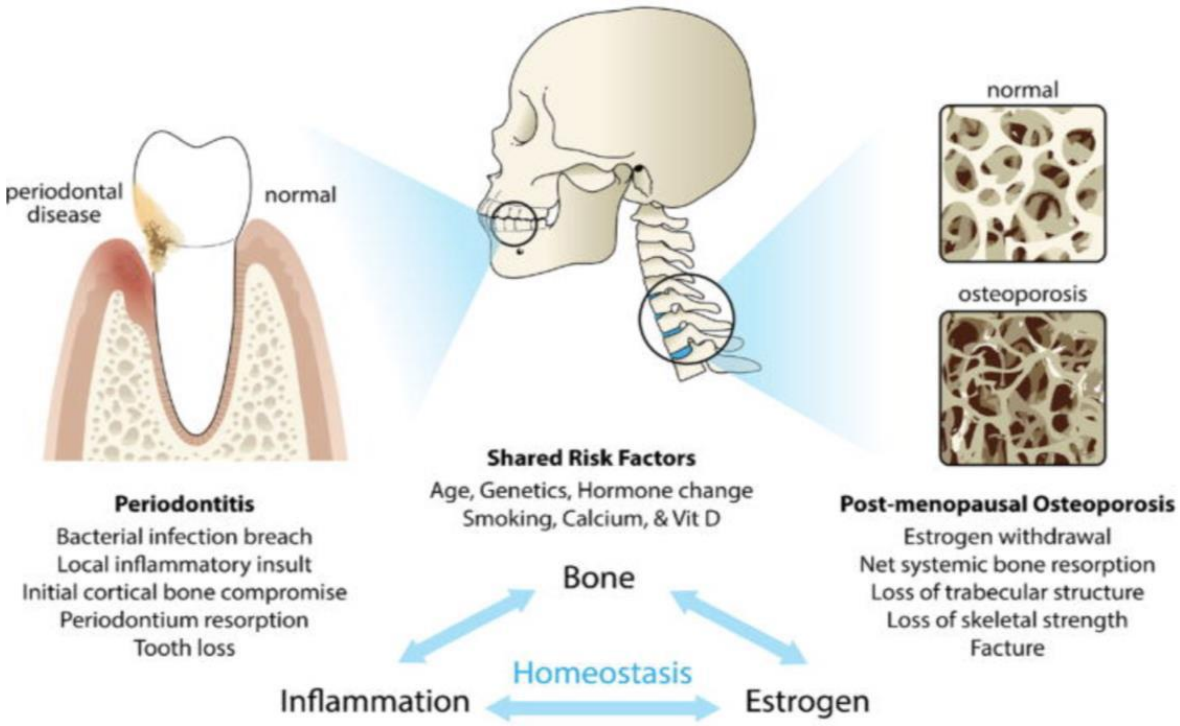


Figure I.31. Periodontal disease / osteoporosis link (Wang & McCauley, 2013)

Confounding factors need to be adjusted, such as a possible oestrogen supplementation treatment. Hormone replacement therapy (HRT) and vitamin D would reduce tooth loss and periodontal incidence. In addition, it would improve mandibular bone density and reduce gingival bleeding (Wang & McCauley, 2013). A study by Nazir (2017) concludes that oestrogen deficiency reduces bone density after menopause, which can lead to alveolar bone loss and possibly loss of teeth. A longitudinal study of 42,171 women in their post menopausal stage showed that the treatment of osteoporosis by hormone therapy with oestrogen led to a reduction in tooth loss (Nazir, 2017). Potential mechanisms have been

proposed but remain insufficient. BMD reduction is a common risk indicator and not a causative factor for periodontal disease (Bansal et al., 2013).

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic degenerative autoimmune inflammatory rheumatism, a disease of the joints that manifests as persistent inflammation. The disease progresses by inflammatory flares of varying duration and intensity and gradually gains new joints. Then, in 20 to 30% of cases, inflammation of the synovial membrane causes progressive breakdown of cartilage and bone in the affected joints and leads to their deformation. In the absence of treatment and in its most serious forms, the disease can be responsible for a handicap and prevent from ensuring daily gestures and professional activity. It is accompanied by systemic inflammatory manifestations, such as dry eyes and mouth (Kim & Suh, 2020).

Like periodontal disease, its etiology is multifactorial but the pathophysiology of RA is still poorly understood. In 2002, a team discovered alpha-enolase (ENO1) as a RA autoantigen. In vitro, ENO1 causes an inflammatory reaction involving monocytes via the TLR-4 receptor (Vittecoq, 2002). Indeed, in RA the presence of antibodies directed against other antibodies (rheumatoid factors) and that of more specific antibodies directed against modified proteins are no longer recognized as proteins of the self by the organism (antibodies directed against citrullinated peptides ACPA). Our immune system produces antibodies to its own citrullinated cyclic peptides that cause RA.

There is a significant association between the prevalence of PD and RA. The pathophysiology of these two diseases is based on an immune response: similar profile of inflammatory cells and pro-inflammatory cytokines. We can say that there is a two-way relationship between RA and chronic periodontitis (Bouchard, 2015).

There are also several common risk factors between these two pathologies: tobacco and genetics. A shared epitope has been identified: HLA-DRB1 as a genetic predisposition in these two diseases (Viatte et al., 2013). The deleterious role of tobacco considerably increases the risk of developing RA, particularly in genetically predisposed subjects (carrying an HLA allele -DRB1). Active smoking increases oxidative stress and causes citrullination of certain proteins. The relationship is dose dependent, in particular the persistence of the risk of RA can persist for 20 years after smoking cessation (Karlson & Deane, 2012).

Periodontal disease promotes joint inflammation through the passage of bacteria and pro-inflammatory cytokines into the blood. In addition, Pg has a specific role in this association. This link is therefore based on two hypotheses: the hypothesis of an immune response through the production of pro-inflammatory cytokines and other common mediators which pass from local to systemic, and the hypothesis of the important role of Pg in the development of RA. According to Huang et al. study (2017), it was suggested that Pg express the peptidyl arginine deiminase (PAD) which catalyzes the citrullination of arginine. Pg is the only prokaryote to have PAD. Citrullinated antigens have been demonstrated in the periodontium of patients with periodontal disease, and circulating antibodies against Pg are correlated with the presence of ACPA. Autoimmunity in RA is characterized by the presence of antibodies to human citrullinated enolase, which would cross-react with citrullinated enolase from Pg.

Pg could therefore be involved in triggering the autoimmune response that precedes the onset of rheumatoid arthritis by increasing the level of citrullinated proteins which could induce the initiation of rheumatoid arthritis by promoting ACPA production.

One of the hallmarks of RA is the persistent synovitis resulting from the immune cell influx into the joints. This pathology is more and more recognized to amount to a more expansive syndrome that contains increased cardiovascular morbidity, psychological

impairment, risk of cancer and osteoporosis (Leech & Bartold, 2015). The past decade has seen the advent of novel therapeutics in the form of both biologic agents and small-molecules. At least as important as these unparalleled advances in drug development has been the introduction of strategic management approaches that have made remission or low disease activity the target of treatment (Calabresi et al., 2018).

Infiltration of the synovial membrane and fluid with leukocytes is defined primarily by cells of the innate immune system. Thus, macrophages, mast cells, natural killer (NK) cells and neutrophils have been described as crucial components of the synovial infiltrate and their effector functions clearly link to disease manifestations. More recently, innate lymphoid cell lineages have been characterized that further extend the contribution of innate effector components to tissue destruction. Synovitis is characterized by wide cellular expression of damage-associated molecular patterns and pathogen-associated molecular patterns that facilitate deregulated activation of these various cell lineages, in the presence of chronic damage but without recourse to specific antigen. Critically, the kinetics of immune function in RA tissues are unlikely to be 'synchronized' by an initiating event and, as such, the normal sequential regulatory homeostasis that is integral to classical innate immune responses in relation to antigen-triggered responses is unlikely to operate in the rheumatoid joint. From the pathological perspective, the net effect of this cellular profile is the generation of tissue-destructive enzymes, reactive oxygen and nitrogen intermediates, prostaglandins and leukotrienes, and a broad range of effector cytokines, outside their normal homeostatic 'on–off' regulatory cycle (Michot et al., 2016).

In this context, effector T cells, together with B cells and other effector cells, form a complex network that promotes the production of pro-inflammatory cytokines, triggering the activation of fibroblast-like synoviocytes and contributing to bone and cartilage lesions. Innate immune cells such as neutrophils and mast cells contribute to the development of synovitis, as well as macrophages, which function by the release of proinflammatory cytokines (such as TNF, IL-1 and IL-6) and inflammatory mediators such as free oxygen radicals, nitrogen intermediates and prostanoids (McInnes et al., 2016).

For many years, the dominant dogma was that the macrophages polarize in the pro-inflammatory phenotype "M1" in the AR, resulting in the production of pro-inflammatory mediators and a reduction in control mechanisms and anti-inflammatory cytokines such as growth factor type TGFβ, IL-4, IL-13 and IL-10. However, phenotypes from synovial macrophages in AR patients are diverse and do not follow a strict M1 or M2 phenotype. It is debatable which of the cytokines have only anti- or pro-inflammatory functions, but it has been observed that they function in a complex network dependent on the specific pathological and tissue context (Chen et al., 2018).

The IL-1 family consists mainly of IL-1α, IL-1β, IL-18 and IL-33. IL-1α is either expressed on the cell surface or is contained within the cell, while IL-1β produces its biological activity by acting on other cells. The action of IL-1 α and IL-1 β may be blocked by an endogenous inhibitor, antagonist of IL-1 receptor (IL-1Ra). Patients with RA show an imbalance between IL-1Ra and IL-1 levels (Dayer, 2018). In many cases a high concentration of IL-1β in plasma and synovial fluid (SF) has been found, which explains various parameters of disease activity, including duration of stiffness.

The cytokine IL-2 is crucial to the function, expansion and survival of regulatory T cells (Treg) and equilibrium in this way is disrupted in Th1 helper cell-mediated autoimmune diseases such as type 1 diabetes mellitus, RA and erythematous systemic lupus. In a study from 2018, patients with RA had elevated levels of anti-IL-2, the authors assuming that they would affect the bioavailability of IL-2 required for Treg homeostasis (Bo et al., 2018).

Interleukin-4 is a major cytokine that promotes the generation of Th2 cells by differentiating naive T cells. Through the positive response loop, IL-4 is further generated by

activated Th2 cells. IL-4Ra is the IL-4 receptor that exists in various forms in the body. Initially, it was reported to be either absent or present in very small amounts in the synovial fluid of patients with AR, but was detected in some RA patients. It is an anti-inflammatory cytokine that prevents the formation of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in synovial tissue and peripheral blood mononuclear cells (PBMCs). In vitro studies have shown that IL-4 reduces bone resorption by having a direct influence on osteoclasts and may also inhibit the production of MMP (Bo et al., 2018).

TNF and IL-6 are considered key points of the synovial system in the RA, which stimulates the formation and subsequent degradation of bone and cartilage and also strongly induce the release of other pro-inflammatory mediators such as IL-1 and stimulatory factor granulocyte-macrophage colonies - GM-CSF. Targeting TNF and IL-6 with neutralizing antibodies or, in the case of IL-6, with Janus kinase inhibitors (JAK), is a strategy now widely used in the treatment of RA, which can effectively suppress synovial inflammation. Furthermore, GM-CSF inhibition has been effective in the treatment of RA in early-stage clinical trials and is currently awaiting approval for clinical use (Baker & Isaacs, 2018).

The role in pathology of IL-6 has been documented in patients with rheumatoid arthritis (RA); to them, there was a tendency of increase in the level of IL-6 in GCF. Thus, one study indicated higher IL-6 serum values in RA patients compared to patients without systemic disease, but comparable to age-type, gender-, smoking-status variables, IL-6 serum levels showing a positive correlation with activity RA. These observations suggest that systemically and locally produced IL-6 may play a role in regulating periodontal inflammation in RA patients (Cosgarea et al., 2019).

Interleukin-17 is of particular interest for the pathogenesis of periodontitis due to its involvement in both inflammation and antimicrobial immunity, some research confirming that IL-17 mediates protection against extracellular pathogens and, together with IL-22 (a cytokine produced also by Th17 and by other IL-17 expressing cells) production of antimicrobial peptides (AMP), which is believed to be protective in periodontitis (Schulz et al., 2019).

In principle, IL-17 is a two-edged paradigm sword for a disease such as periodontitis that is initiated by bacteria, although tissue damage is caused by the host's response. Therefore, the biological properties of IL-17 make it difficult to estimate its role in inflammatory diseases with a polymicrobial etiology. It is possible that IL-17 exerts both protective and destructive effects as suggested in distinct mouse models, although signalling of the IL-17 receptor may turn an acute protective inflammatory response into chronic immunopathological events (Abusleme & Moutsopoulos, 2017).

P. gingivalis stimulation of PBMC in healthy volunteers led to an increase in IL-17 production in CD3 + cells and an increase in IL-23 production in macrophages. Furthermore, the isolated lipopolysaccharide from *P. gingivalis* has been shown to induce IL-17 and IL-23 production from human periodontal ligation cells, while external membrane proteins can stimulate mRNA expression for IL-17 in PBMC isolated from patients with gingivitis or periodontal disease (Hajishengallis et al., 2017).

In part, the suppression of Th1 cell-mediated immunity by *P. gingivalis* could be attributed to its ability to inhibit the production of chemokine in the gingival epithelial cells responsible for Th1 recruitment. Generally, *P. gingivalis* has an arsenal of virulence factors by which it can manipulate specific and non-specific immune cells to initiate an IL-17 orchestrated rich inflammatory response (Potempa et al., 2017).

Importantly, the presence of *P. gingivalis* in subgingival biofilm was associated with increased IL-17 levels in crevicular fluid in periodontitis (Mikuls et al., 2014).

Continuing advancement in scientific methodology, including high throughput analysis techniques, is enabling studies on genomic variations and gene expression patterns in

periodontal disease and rheumatoid arthritis.

Whole-genome microarrays and RNA sequencing will be valuable tools for identifying genetic and biological markers of increased susceptibility to both pathologies shedding light into the possible implications and interdependencies. In recent years, both transcriptome studies and a genome-wide association study have been performed on periodontitis cohorts (Santiago-Rodriguez et al., 2015).

The massive amounts of data generated by such studies require painstaking analyses to yield biologically significant results, but the capacity for identification of novel mediators involved in the pathogenesis of both diseases is promising (Martu et al., 2018).

This research direction has been materialized by publishing the following paper:
Martu A, Solomon SM*, Sufaru IG, Jelihovschi I, Martu S, Rezus E, Surdu AE, Onea RM, Grecu GP, Foia L. Study on the prevalence of periodontopathogenic bacteria in serum and subgingival bacterial plaque in patients with rheumatoid arthritis. Rev. Chim (Bucharest) 2017; 68(8): 1946-1950**.**
http://www.revistadechimie.ro/pdf/56%20MARTU%208%2017.pdf

I.6.3.1 The prevalence of parodontopathogenic bacteria in serum and subgingival bacterial plaque in patients with rheumatoid arthritis

Aim of the study

We conducted a study with the main purpose to detect bacterial periodontal DNA in the subgingival dental plaque and serum in patients affected by rheumatoid arthritis and periodontitis.

Materials and method

The study group included 19 patients with periodontitis and refractory rheumatoid arthritis despite intensive treatment with anti-rheumatic diseases (DMARD) (methotrexate, sulfasalazine, leflunomide and chloroquine).

The patients completed a health questionnaire that included information on systemic health and oral diseases. Written and informed consent from patients was obtained prior to clinical examination in accordance with the ethical principles of the Helsinki Declaration. The patients included in this study were males and females over 18 years of age who had persistent rheumatoid arthritis with synovial fluid effusion on their knees without other systemic diseases and affected by periodontitis.

Patients who received antibiotic therapy at least three months prior to the study, those who received pre-treatment for periodontal disease, pregnant women and nursing mothers were excluded from the study. All patients were treated with rheumatic DMARD-modifying medication, most of them also benefited from non-steroidal anti-inflammatory medication and low-dose steroids.

The diagnosis of periodontitis was determined by measuring the depth of the periodontal pocket and the clinical index of loss of attachment. These indices were obtained using a periodontal probe, the Merrit B (Hu-Friedy) probe graduated in millimetres (0-10 mm).

Ten millilitres of peripheral blood from the cubital vein were collected from each patient and placed inside vacuum tubes and citrate medium. They were centrifuged at 275 g for 8 min to obtain the serum.

The subgingival dental plaque has always been collected after obtaining blood samples to avoid transient bacteraemia that may influence the presence of different bacterial species in the serum. After cleaning the crown of the tooth with a sterile sponge, a subgingival plate

was collected with a Gracey curette from the vestibular, medial, palatinal, and distal sulcus. This was placed in an Eppendorf tube with 1 ml of phosphate buffered saline (PBS). The subgingival plaque was harvested from the first upper molar in quadrant I, from the lower central incisor in quadrant III, and from the lower premolar in quadrant IV as they present the most affected areas of periodontitis, and the possibility of obtaining the subgingival dental plaque was more feasible.

The sample from each tooth was placed in a different tube; the sample was collected 2 hours after the last meal and dental brushing. The subgingival plate and peripheral blood were transported with ice and stored at -40°C until polymerase chain reaction (PCR) and microbiological assessments were performed. All samples were processed under aseptic requirements to prevent contamination of both the environment and the DNA extraction method for PCR assays.

Positive controls were included in each PCR set using DNA from the following bacterial strains: *P. gingivalis* (ATCC 33277 and HG1691), *T. forsythia* (ATCC 43037), *Prevotella intermedia* (ATCC 25611), *Aggregatibacter actinomycetemcomitans* (ATCC 29523 and HK1651), *P. nigrescens* (ATCC 25261) and *T. denticola* (ATCC 35405). A control (negative) test sample was also included in each PCR set containing a sample only with deionized water (instead of a patient sample) to know if non-specific products were amplified.

For the statistical analysis, the ESPE SS 12 package was used, and the α value was set to 0.05. Normally distributed variables were reported as standard deviations. The Chi-Square and Fisher tests with Anova were used to compare the data. To determine the statistical differences in the periodontal bacterial DNA detection near the dental plaque and serum, Fisher's exact test was also used and the statistical significance was established at $p < 0.05$.

Results

The mean age of patients was 55.7 (± 15.8) years, with a range of 21 to 88 years of age. Sixteen patients (84.2%) were females. The time course of rheumatoid arthritis was 8.71 (± 5.99) years, with a range of 0.5-20 years from the initial clinical diagnosis of rheumatoid arthritis. The most common type of periodontitis detected was the chronic form found in 18 subjects (94.7%); the aggressive form was present in only one subject (5.3%) of the 19 subjects. The severe phase of chronic periodontitis was more commonly diagnosed (42.2%) than the moderate and mild stages of chronic periodontitis (36.8% and 21.1%, respectively). The average overall depth of the pocket was 3.9 (± 0.81) mm, but given the deepest depth of the pocket of each tooth, the average was 4.2 (± 0.79) mm. In terms of loss of attachment, the average was 3.63 (± 0.90) mm, the upper molars being the most affected teeth, with an average of 3.85 (± 0.83) mm. Subjects presented 63.8% of teeth present in the arcade; lower molars were the most frequently absent teeth (46%) (Table I.28). The DNA of parodontopathogenic bacteria was detected in 100% of the subgingival plate samples, and in serum samples it was identified in 84.2% of cases.

Regarding the number of identified bacteria species, 4.05 different bacterial species were detected in subgingival samples and 1.19 species were detected in serum samples.

The most commonly found species in subgingival samples were *P. Intermedia* (100%), *T. denticola* (84.2%) and *P. gingivalis* (78.9%). In serum samples, the most frequently detected species were *P. Intermedia* (89.4% and 73.6% respectively) and *P. gingivalis* (57.8% and 42.1%, respectively) (Table I.29).

The less common species detected was *A. actinomycetemcomitans* (15.7%). Comparing the two types of biological samples we can see that *A. actinomycetemcomitans* and *P. gingivalis* did not show significant statistical differences in the samples. On the other hand, *P. Intermedia, T. forsythia, P. nigrescens* and *T. denticola* statistically showed a significant

difference (Table I.29). The most commonly found species in all types of serum samples were *P. intermedia* and *P. gingivalis* (63.1% and 36.8%, respectively).

There was no negative topic for *P. intermedia* (Table I.30). The most common species absent in all samples was *A. actinomycetemcomitans* (78.9%).

Table I.28. Dental parameters on number of patients

	Mean value	**Standard Deviation (SD)**	**Interval**
Pocket depth (mm)	3.9	0.81	2.6-5.7
Present teeth (n)	17.89 (63.8)	8.93	3-27
Superior anterior teeth (6)	4.10 (68.3)	2.33	0-6
Inferior anterior teeth (6)	4.52 (75.3)	1.92	1-6
Superior premolars (4)	2.36 (59)	1.60	0-4
Inferior premolars (4)	2.94 (73.5)	1.31	0-4
Superior molars (4)	2.10 (52.5)	1.37	0-4
Inferior molars (4)	1.84 (46)	1.57	0-4
n= 19.			

Table I.29. Subgingival and serum periodontal bacterial DNA

	Dental plaque		Serum		**p-value**
	Frequency	%	Frequeny	%	
Prevotella intermedia	19	100	14	73.6	**0.0453**
Tanerella forsythia	10	52.6	6	31.5	**0.0203**
Prevotella nigrescens	13	68.4	0	0	**<0.0001**
Aggregatibacter actinomycetemcomitans	4	21.0	0	0	**0.1204**
Porphyromonas gingivalis	15	78.9	8	42.1	**0.0674**
Treponema denticola	**16**	**84.2**	**4**	**21**	**0.0004**

Table I.30. Periodontal bacterial DNA detected in different combinations

Bacteria	Negative subgingival and serum samples (absence of bacterial DNA) (no. of samples/percentage values)	Positive subgingival and serum samples (presence of bacterial DNA) (no. of samples/percentage values)	**Positive subgingival samples (presence of bacterial DNA) (no. of samples/percentage values)**
Prevotella intermedia	0 (0)	2 (10.5)	**0 (0)**
Tannerella forsythia	9 (47.3)	4 (21.0)	**4 (21.0)**
Prevotella nigrescens	5 (26.3)	0 (0)	**9 (47.3)**
Aggregatibacter actinomycetemcomitns	15 (78.9)	0 (0)	**1 (5.2)**
Porphyromonas gingivalis	4 (21.0)	1 (5.2)	**3 (15.78)**
Treponema denticola	**3(15.78)**	**1 (5.2)**	**9 (47.3)**

Discussion

Both rheumatoid arthritis and periodontal disease share similar immunopathological mechanisms, because the virulence factors produced by periodontal bacteria produce an immune response that is mediated by neutrophils, monocytes, B and T lymphocytes which lead to an increase in the release level of prostaglandins that stimulate osteoclastic activity and lead to bone erosion, similar to the mechanism involved in rheumatoid arthritis.

As part o the inflammatory link between periodontitis and RA, certain inflammatory mediators such as IL-1b, MMP-8 and TNF-a are elevated in the inflamed joints and serum of patients with RA and as a consequence, is possible that persons with RA or other inflammatory arthritis could have increased levels of these potential biomarkers in their saliva.

In a previous study (Boatca et al., 2015), we observed that the RA group had clinical measures less severe than the periodontitis group despite the salivary IL-1b levels being higher in the RA group, suggesting that salivary IL-1b was elevated due to systemic inflammation. IL-1b levels showed a positive correlation with %PD sites >4 and >5mm in the periodontitis group.

Statistical analysis of TNF-α results in the three groups of patients showed a significant difference between the group of patients with slightly chronic periodontitis and RA and patients with chronic periodontitis group without RA.

Looking at the average values of TNF-α note that in the group of patients with chronic periodontitis and RA, values are almost double the control group of patients. In the group of patients with chronic periodontitis periodontitis and TNF-α RA values were the highest recorded maximum value of 3.5ng / ml.

A common microbial pathogen involved in both pathologies, rheumatoid arthritis and periodontal disease is *P. gingivalis*. Protein deminination is facilitated by a PAD of the peptidyl enzyme arginine deiminase, an enzyme released by *P. gingivalis*. This, in turn, causes a pro-inflammatory response to citrulline proteins. This biochemical reaction is a vital factor in the progression of rheumatoid arthritis.

There have been studies exploring associations between periodontal bacteria and rheumatoid arthritis. They are mainly concentrated on detecting antibodies against various bacteria, especially in serum. In a case-control study, serum antibodies against periodontal bacteria and subsequently causing disease were identified more frequently in subjects affected by rheumatoid arthritis and periodontitis than in control subjects (Ogrendik et al., 2005).

In any case, it is important to bear in mind that the detection of periodontal bacterial DNA in patients with rheumatoid arthritis is more important than the detection of antibodies because it suggests the transport of bacterial DNA from periodontal infections to the joints of patients with rheumatoid arthritis.

In this study, only 19 patients were included due to the difficulty of finding patients who met the inclusion criteria. Patients with refractory rheumatoid arthritis treated with DMARD-modifying disease of the periodontal disease were selected because this condition was necessary to obtain a serum sample with significant characteristics.

Most patients were female (84.2%), and was consistent with the information that rheumatoid arthritis affects women more than men. The most common forms of periodontitis found in this study were those of chronic form; this may be due to the age of the included patients.

In the present study, periodontal bacterial DNA was detected in 100% of subgingival plaque samples and 84.2% in serum samples. Regarding the number of bacterial species detected, a large number (4.05) of bacterial species was identified in the subgingival plate,

followed by serum (1.19). The fact that there is a lower presence of serum bacterial DNA can be explained by its dilution from the blood stream by renal filtration. This data is consistent with other studies where bacterial DNA has been detected by DNA-DNA chess hybridization resulting in 100% positive serum samples.

Several species commonly identified in serum were *P. intermedia, P. gingivalis* and *T. denticola*; two of them belong to the red complex, which is associated with destructive diseases. On the other hand, *A. actinomycetemcomitans*, mainly responsible for aggressive periodontitis, was less frequently detected. The reason could be that only one patient was affected by aggressive periodontitis.

A. actinomycetemcomitans and *P. gingivalis* did not show statistically significant differences between samples. This finding suggests that it could be an association because the same bacteria species detected in the serum were present in bacterial plaque samples. On the other hand, there were statistical differences between samples for *P. intermedia, T. forsythia, P. nigrescens* and *T. denticola*. In addition, *P. intermedia* and *P. gingivalis* were the most frequently detected species in the three sample sites.

The tooth associated microflora has been extensively studied. The presence of *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans* poses an increased risk for periodontitis. The microbiota of healthy periodontal sites and those affected by the disease have been shown to differ from one another. A small number of microorganisms and fewer morphological types can be found in the healthy gingival sulcus. Affected sites have a complex microflora with a high proportion of gram-negative anaerobic microorganisms.

The microbiota of healthy periodontal sites and that of the affected sites have been shown to differ from one another. Reduced numbers of microorganisms and fewer morphological types can be found in healthy gingival sulcus. Affected sites have a complex microflora, with a high proportion of anaerobic gram-negative bacteria.

It is important to note that patients who were positive for any bacteria in the serum were also positive when detected in the subgingival plaque. *P. gingivalis* produces a microbial enzyme, peptidyl deaminase arginine (PAD), which is the human equivalent of this enzyme and which has been associated as a susceptible factor for rheumatoid arthritis, since the antigens generated by arginine peptidyl deaminase lead to the production of rheumatoid factor and local inflammation (Potempa et al., 2017).

We therefore suggest that bacterial DNA can play a role in the pathogenesis of rheumatic diseases. The transport of DNA from periodontal pockets to the joints could be the free DNA. This information can be valuable for future studies to elucidate whether periodontal pathogenic DNA might be a possible trigger for rheumatoid arthritis development.

Periodontal bacterial DNA was detected in the subgingival plaque of patients with rheumatoid arthritis. It is therefore suggested that periodontal bacterial DNA plays a major pathological role in the severity of rheumatoid arthritis. *P. intermedia, T. forsythia*, and *P. gingivalis* were the most commonly found species in the subgingival dental plaque, the corresponding and predominant bacteria in the red complex, which is involved in the destruction of the periodontal bone.

Conclusions

Patients with rheumatoid arthritis contain serum levels of antibodies to oral pathogens, which are common to red complex organisms, namely *Porphyromonas gingivalis, Tannerella forsythia* and *Prevotella intermedia.*

The data obtained in this study provides evidence to demonstrate the existence of a link between rheumatoid arthritis and periodontal disease.

Upcoming breakthroughs in the understanding and treatment of these pathologies need not be derived only from periodontitis or arthritis focused research. Much is to be gained from research progress in other chronic inflammatory conditions.

Studies are ongoing to evaluate the role of other proinflammatory cytokines in the pathophysiology of similar conditions, such as lupus, Crohn's disease, Alzheimer's and irritable bowel disease, and to develop antibodies which specifically target these cytokines for novel future treatment strategies. These mechanisms give particular interest to the links existing between these two pathologies and to potential future treatments.

I.6.4 The relationship between chronic viral hepatitis and periodontal disease

State of the art in the periodontal disease - chronic viral hepatitis relationship

Chronic viral hepatitis C (CHC) occurs as a consequence of the infection with the hepatitis C virus (HCV) that uses hepatic or peripheral blood cells for replication. The spread of the infection is considered a global health issue, as it interests more than 200 million people worldwide, and is particularly difficult to combat, because during its initial stages, the disease has no symptoms. As a result, infected persons may be unaware and prone to infect others (Wedemeyer, 2015). After the acute stage of infection, most patients will develop a chronic inflammation of the hepatic tissue. Gradually, the hepatic function is impaired, as the hepatic tissue is replaced with fibrotic tissue (Bataller & Brenner, 2005) and liver cirrhosis occurs. Chronic hepatitis C patients can also develop other life-threatening complications, such as hepatocellular carcinoma (Gheonea et al., 2014). Being a chronic inflammatory condition, CHC could exhibit some correlations and interactions with PD, possibly via the pro-inflammatory mediators that are discharged into the blood flow of HCV patients (Gheorghe et al., 2018). Natural killer cells (NK), which have TNF production capabilities, play an important role in the immunological pathogenesis of CHC (Yoon et al., 2016). Blood levels of certain cytokines, such as IL-18 and IL-33, are used as an indicator of CHC disease activity and severity, as these patients have been shown to exhibit increased serological levels of these interleukins (Wang et al., 2012). Elevated levels of interleukin-1α have also been found in serum samples of chronic hepatitis C patients, and have been directly associated with the severity of the disease (Tawfik et al., 2018). Furthermore, some pro-inflammatory mediators, such as interleukin-1β, have been shown to promote liver inflammatory processes in chronic hepatitis C patients (Negash et al., 2013), expressing increased serum levels in these patients.

This research direction has been materialized by publishing the following paper:
Surlin P, Gheorghe DN, Popescu DM, Martu MA, Solomon S, Roman A, Lazar L, Stratul SI, Rusu D, Foia L, Boldeanu MV, Boldeanu L, Danilescu M, Rogoveanu I. Interleukin-1α and -1β assessment in the gingival crevicular fluid of periodontal patients with chronic hepatitis C. Exp Ther Med. 2020; 20(3): 2381-2386.
https://doi.org/10.3892/etm.2020.8906

I.6.4.1 Interleukin-1α and -1β assessment in the gingival crevicular fluid of periodontal patients with chronic hepatitis C

Aim of the study

The study objective was to analyse the impact that chronic hepatitis C could have on the inflammatory status of patients with chronic periodontal disease, who also suffer from HCV infection, by assessing the GCF levels of interleukin-1α (IL-1α) and interleukin-1β (IL-1β) in these patients, and comparing the results to those of systemically healthy

periodontal patients. Higher levels of these particular interleukins, which have considerable implications in the pathogenic processes of both PD and CHC, could imply that chronic hepatitis C acts as an additional risk factor for PD and that it has a negative impact on the inflammatory status of periodontal patients, assessed by interleukin detection.

Materials and method

For each patient the following periodontal parameters were recorded: the number of teeth with periodontal pockets deeper than 4 mm (AT), the maximum periodontal probing depth (MD) and the number of existing/remnant teeth (RT). Only patients having at least two periodontal pockets in two distinct teeth, with minimum 5 mm probing depth, were included in the study. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

Patients suffering from asymptomatic chronic hepatitis C were included in the study, the diagnosis being based on blood tests results regarding the aspartate transaminase (AST) and alanine transaminase (ALT) serum levels. The age of the HCV infection diagnosis was also recorded for the chronic hepatitis C patients.

Patients not suffering from systemic diseases, and who were diagnosed with chronic periodontal disease, through the periodontal examination, were included in the study, as part of the periodontally affected, but systemically healthy group.

The study also comprised a group of control patients, periodontally and systemically healthy, who were referred to the Department of Periodontology for pre-orthodontic periodontal evaluation.

For the unbiased evaluation of their inflammatory status, patients were excluded from the study if they met at least one of the following exclusion criteria: i) Anti-inflammatory treatment in the last month; ii) antibiotic treatment in the last 3 months; iii) pregnancy; iv) decompensated or symptomatic chronic hepatitis C; v) current smoker and vi) any diagnosed systemic condition other than chronic hepatitis C.

After applying the inclusion and exclusion criteria, three groups of patients were set up for the study: i) P group, 13 patients with chronic periodontal disease and no systemic disease (6 male and 7 female, aged between 40-58 years); ii) PH group, 11 patients with chronic periodontal disease and asymptomatic chronic hepatitis C (8 male and 3 female, aged between 36-62 years); iii) H group, 11 control patients with no systemic and periodontal disease (7 male and 4 female, aged between 31-43 years).

The enzyme-linked immunosorbent assay (ELISA) was used for qualitative and quantitative assessment of IL-1α and IL-1β in the GCF samples. Commercial detection kits designed for the micro-detection of the two cytokines were used (Quantikine; R&D Systems) in accordance with the manufacturer's indications and prescribed method. In order to decrease optical imperfections on the reading plate, reading was performed at 450 with 540 nm corrections. The obtained results were expressed in pg/ml.

Data was statistically analysed with Microsoft Office Excel Data Analysis tool kit software (Microsoft Corporation), the resulted mean values and standard deviations being used as primary data (mean ± SD). The statistical significance of the results was assessed using the Mann-Whitney U test ($P<0.05$) for comparison between the different study groups. Correlations between statistical and clinical data were made using Pearsons's test.

Results

The GCF levels of pro-inflammatory cytokines, for both IL-1α and IL-1β, were the highest for the PH group, followed by the P group, while the lowest were those of the H group (Fig. I.32).

Statistically significant differences ($P<0.05$) were recorded for GCF IL-1α levels,

between the PH and P groups (the levels for the PH group being 1.8-fold higher than that for P group), between the PH and H groups (the levels for the PH group being 6.9-fold higher than that for the H group) and between P and H groups (the levels for the P group being 3.8-fold higher than that for the H group) (Fig. I.32).

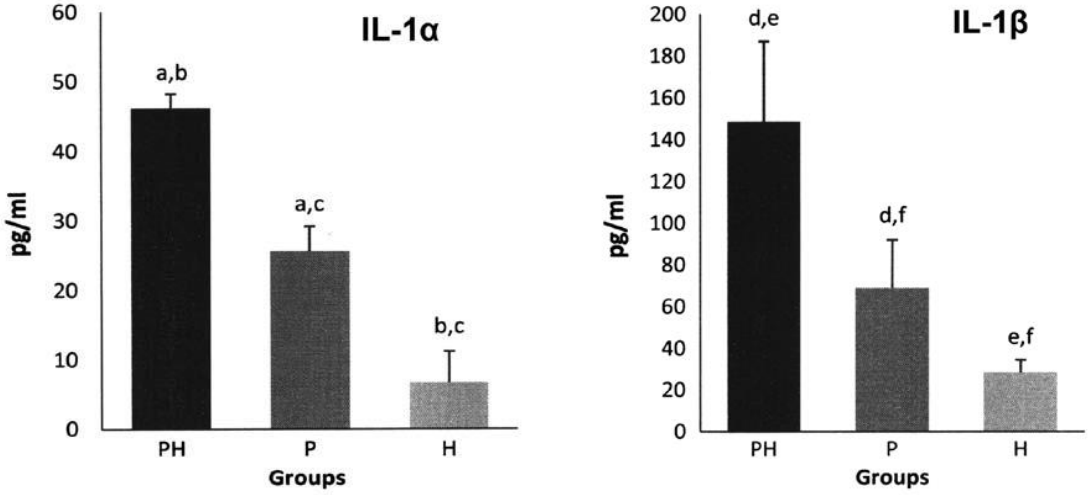


Figure I.32. GCF levels of IL-1α and 1β for each group (pg/ml). a) Statistically significant difference between PH and P groups for IL-1α GCF levels; b) statistically significant difference between PH and H groups for IL-1α GCF levels; c) statistically significant difference between P and H groups for IL-1α GCF levels. GCF, gingival crevicular fluid; IL-1α, interleukin-1α, IL-1β, interleukin-1β; d) Statistically significant difference between PH and P groups for IL-1β GCF levels; e) statistically significant difference between PH and H groups for IL-1β GCF levels; f) statistically significant difference between P and H groups for IL-1β GCF levels. GCF, gingival crevicular fluid; IL-1β, interleukin-1β.

Table I.31. Correlations between the assessed clinical and immunologic parameters.

Index	PH Group		P Group		H Group	
	IL-1α	IL-1β	IL-1α	IL-1β	IL-1α	IL-1β
RT r p	-0.44 0.17	-0.29 0.37	-0.42 0.13	-0.3 0.3	-0.37 0.25	-0.8 0.02
MD r p	0.23 0.48	0.35 0.26	0.21 0.42	0.21 0.41		
AT r p	0.32 0.33	0.25 0.37	0.28 0.31	0.68 0.01		
DG r p	0.23 0.49	0.29 0.38				
RT, number of existing/remnant teeth; MD, maximum probing depth; AT, number of teeth with periodontal pockets >4 mm; DG, age of HCV infection diagnosis; r, Pearson's r for correlation strength; p, statistical significance index; IL, interleukin.						

The average GCF levels of IL-1β expressed statistically significant differences ($P<0.05$) between the PH and P groups (the levels for the PH group being 2.1-fold higher than that of the P group), between the PH and H groups (the levels for the PH group being 5.1-fold higher than that for the H group) and between the P and H groups (the levels for the P group being 2.41-fold higher than that for the H group) (Fig. I.32). In P and PH patients, the levels of

GCF cytokines expressed correlations with certain parameters of periodontal status (Table I.31). For the PH group, the MD was positively correlated to the GCF levels of both IL-1α and IL-1β. For the P group, a moderate positive correlation was found between the MD parameter and the level of GCF cytokines. AT did not express significant statistical difference between the PH and P groups. No significant correlations were found between this periodontal parameter and the GCF levels of IL-1α or IL-1β. RT was significantly different between the PH and P groups. This parameter expressed negative correlations with the GCF levels of both IL-1α and IL-1β.

For the PH group, the age of the HCV infection diagnosis expressed a slight positive correlation with the GCF levels of IL-1α and IL-1β.

Discussion

Elevated gingival fluid levels for IL-1, found in periodontal patients, compared to healthy controls, have been shown to decrease after periodontal treatment (Reis et al., 2014), a fact which further supports their important role in periodontal disease development. The results of the present study expressed a statistically significant difference between the GCF cytokine levels of the PH and P groups. Since patients in both groups were diagnosed with periodontal disease, in similar degrees of severity and evolution, the higher GCF cytokines levels in periodontitis patients with chronic hepatitis C, could be explained by the additional chronic hepatic inflammation that these patients manifest. This fact can impact the intensity of the inflammatory periodontal reaction as well. Moreover, the GCF cytokine level was significantly different between P and H groups and between PH and H groups, suggesting the reliability of cytokines as indicators of the inflammatory status.

In chronic periodontitis patients, the levels of GCF cytokines expressed a positive correlation to the degree of periodontal inflammation. In the present study, the clinical indicators used for the assessment of the periodontal status (MD, AT, RT) were correlated to the GCF levels of IL-1α and IL-1β in periodontal patients with chronic hepatitis C. There was a moderate positive correlation also between these clinical parameters in periodontal patients with no systemic condition and the GCF cytokine levels, suggesting the additional effect that systemic chronic inflammation can have on the periodontal status of such patients. The number of remnant teeth was statistically significantly different between the three groups and in a negative correlation with the GCF cytokine levels, emphasizing the important impact that severe chronic inflammation has on the dental and periodontal history of the patient, frequently resulting in the loss of teeth, as a consequence of periodontal disease. Due to the reduced number of participating patients in the study, consequent to the numerous exclusion criteria, required in order to follow a precise and reliable scientific method, the identified correlations generally lacked statistical significance, despite their moderate correlation strength. This issue motivates the extension of the study design on a broader cohort of participating patients.

The number of teeth with periodontal pockets deeper than 4 mm (AT) was not statistically different between the PH and P groups and it did not correlate with the GCF cytokine levels. However, the average maximum periodontal pocket depth (MD) was statistically different between the PH and P groups and expressed a positive correlation to the GCF levels of both IL-1α and IL-1β. This fact could imply that hepatic chronic inflammation could have a significant impact on the severity of periodontal disease and a less important one on its extent, as in the number of affected teeth.

In this study, there was a moderate positive correlation between the GCF levels of cytokines of the PH group patients and the age of their HCV infection diagnosis. This correlation suggests that, as the chronic hepatic inflammatory reaction progresses, it has an increasingly important negative impact on the inflammatory periodontal status of CHC

patients, who also suffer from periodontal disease. Thus, periodontal and hepatic chronic inflammatory reactions could influence one another, as they may be fuelled by the same pro-inflammatory cytokines, i.e. IL-1α and IL-1β. These correlations suggest the important negative impact that hepatic chronic inflammation has on the periodontal status regarding the intensity of the periodontal inflammatory reaction.

In CHC patients, elevated levels of IL-1α and IL-1β were showed in serum samples, in previous studies (Tawfik et al., 2018). Levels of certain cytokines were significantly elevated when the CHC patients were also suffering from diabetes (Jia et al., 2002). Furthermore, the incidence of type Ⅱ diabetes among CHC patients is higher than that of the general population (Drazilova et al., 2018). One explanation for this correlation can be found in the impact of chronically elevated systemic IL-1β levels on general homeostasis (Gao et al., 2014). It appears that, cellular insulin resistance is closely linked to elevated levels of cytokines, which are common in CHC patients (Kukla et al., 2015). In the hepatic tissue of affected patients, insulin receptor substrate has been reported to be impaired, together with the disruption of normal insulin signalling pathways. Increased cytokine expression (such as TNF-α) could also contribute to the onset of insulin resistance in CHC patients (Bose & Ray, 2014). Consequently, the cells become insensitive to insulin action and glucose is unable to enter inside them, in order to be metabolised. Thus, chronic hyperglycaemia occurs, the most important pathologic manifestation of diabetes mellitus. Interestingly, insulin resistance has also been associated with PD, while elevated levels of IL-1β have often been recorded in chronic periodontitis patients (Demmer et al., 2012). The interactions between PD and liver diseases, including CHC, could be mediated by either bacterial elements, pro-inflammatory cytokines or oxidative stress (Han et al., 2016). Elevated GCF levels of IL-1α and IL-1β could explain the pathogenic mechanism that connects PD and CHC. By changing the general homeostasis, the chronic inflammatory status caused by PD and CHC, inflicts a modified cellular response. Patients with CHC and PD exhibit elevated cytokine profiles, suggesting the bi-directional impact that chronic hepatic inflammation and periodontal disease could manifest on each other. Insulin resistance could be one of the probable pathogenic mechanisms to mediate the PD-CHC relationship (Han et al., 2016), motivating extended future research on the matter.

Conclusions

Elevated levels of IL-1α and IL-1β, which have considerable implications in the pathogenic processes of both PD and CHC, could imply that chronic hepatitis C has a negative impact on the inflammatory status of periodontal patients, as assessed by interleukin detection.

CHAPTER II

COMPLEX TREATMENT OF PERIODONTALLY AFFECTED PATIENTS

II.1 Effects of root planing with power-driven reciprocating instruments

State of the art in mechanical subgingival debridement

The periodontal disease is an infectious disease, with important inflammatory characteristics, with a high prevalence among the world populations. A new classification system for the periodontal diseases was proposed which included 4 disease stages of periodontitis to describe extent and complexity of the disease and 3 grades to reflect biological features and risk for further progression. The grading system would also implement the analysis of potentially poorer outcomes of treatment (Papapanou et al., 2018).

The determinant agent in the onset and in the evolution of the periodontal disease is represented by the periodontal pathogenic bacteria. They are gathered in a special form of community named biofilm; the main characteristic of the biofilm is cell adhesion which is strictly related to the contact between microorganisms and non-exfoliative surfaces. Another specific characteristic of bacterial biofilm is the presence of the ‘‘matrix of extracellular polymeric substance’’, which contains polysaccharides, proteins and DNA, whose formation is a consequence of the metabolism of the microbial community forming the biofilm. Biofilm organization provides bacteria cells with a strong resistance against pharmacological and chemical therapies. This resistance could be explained by the impermeability of the matrix, by the negative influence of the internal biofilm environment on antibacterial agent activity, such as oxygen gradients, and by an altered growth rate of biofilm organisms (Hoiby et al., 2010; Bjarnsholt et al., 2010).

The primary goal of periodontal therapy is to preserve the natural dentition, by arresting the chronic inflammatory process that results in loss of periodontal attachment and alveolar bone and formation of periodontal pockets. The current understanding on the aetiology and pathogenesis of periodontitis acknowledges that this disease is the result of a complex interplay of bacterial aggression and host response, modified by behavioural and systemic risk factors. Only therapies achieving the mechanical disruption of sub-gingival biofilms have proven successful and, hence, periodontal health can be maintained only provided there is adequate plaque control (Graziani et al., 2018). Mechanical root debridement is the cornerstone of cause-related periodontal therapy and it is aimed at removal of sub-gingival biofilm and calculus, which together with the patient’s oral hygiene practices will prevent bacterial re-colonization and formation of supra-gingival biofilms.

The scaling and root planning (SRP) represents the gold-standard of the periodontal therapy in periodontitis patients. The ability of the fibroblast to adhere to the root surface (which is essential for the periodontal regeneration) depends on the existence of a clean, non-toxic surface, free from bacterial plaque and calculus. Therefore, the purpose of the SRP consists in obtaining a smooth and clean surface, biologically acceptable. Its efficacy is well documented in systematic and narrative reviews (Aimetti, 2014; Geisinger et al., 2019) by the demonstration of gains in clinical attachment levels, reductions in probing pocket depths, and in the frequency of bleeding on probing.

Different instruments may be appropriate for subgingival instrumentation, demonstrating differences in the removal of soft and hard subgingival deposits. Traditionally,

SRP has been performed with curettes, which have been modified by changing the shape of the instrument or the active tip (After Five and Mini-Five curettes) to optimize their instrumentation efficacy in areas of difficult access.

Similarly, power-driven instrument devices using sonic or ultrasonic technologies have improved their outcome performance and modified their application tips so as to improve their capacity of sub-gingival plaque and calculus removal. Ultrasonic instruments have been used in periodontal treatments since the 1960s (Green and Sanderson, 1965). The advantages of using the US systems include the exclusion of the sharpening of the active part, a lower working time and fatigue during instrumentation, when compared to the manual sub-gingival SRP (Zucchelli et al., 2009); the application of US seems to be associated with a number of hazards that need to be avoided, to ensure the safety of operators and patients in the dental practice (Trenter and Walmsley, 2003).

Figure II.1. Per-io-tor handpiece (www.dentatus.com)

Apart from the mechanical debridement, US present also a bactericide effect by the cavitation phenomena; acoustic cavitation is defined as ‘‘the formation of tiny gas bubbles in the tissues as the result of US vibration’’ (Macon et al., 2018). These bubbles, formed around the tip of the US insert, break, generating shockwaves which promote the breaking of the biofilm organisation and the dispersion of the bacteria. In the presence of blood, the cavitation effect can exert a trombogenic result, reducing the bleeding.

The classical US systems allowed an instrumentation of periodontal pockets up to 3mm in depth; the development of new inserts excluded this limitation, nowadays the depth of the periodontal instrumentation by US rising to up to 10mm. In clinical practice, SRP treatment often includes a combination of instruments, manual and mechanized. A very popular system in the Western countries but still lacking in Romania is the reciprocating system Periotor, developed by Axelsson in 1992 (Figure II.1). The set includes different types of inserts, adapted to plane but also to less accessible areas (Figure II.2)

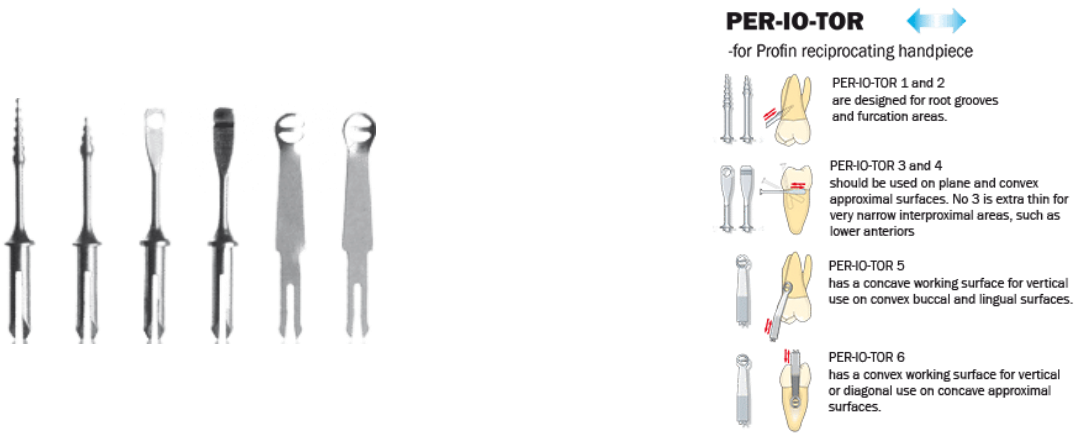


Figure II.2. Per-io-tor inserts (www.dentatus.com)

The previous instrumentation principles supported the need of the removal of all infected periodontal tissues, including the cementum with endotoxins derived from the bacterial cell membranes (Smart et al., 1990). However, British and Scandinavian workers have suggested that the bulk of the endotoxin resides in the sub-gingival plaque, with only small amounts penetrating superficially into the cemental surface (Obeid and Bercy, 2005). Therefore, removing the cementum becomes an unjustified and, moreover, an un-recommended act because it can lead to complications (the denudation of the dentin is associated to dentin hypersensitivity to different physical and chemical stimuli, to root carious lesions). Thus there is a risk that too aggressive instrumentation leads to undue root substance removal.

A clean smooth cementum surface is extremely important for the healing of periodontitis and for the regeneration of the periodontal tissues. The thickness of cementum is only of 0,03 - 0,1 mm in the coronal 1/3 of the root. Therefore 10-20 strokes with a curette can generate the complete removal of the root cementum, leading to undesired complications such as the invasion of subgingival microflora in the dentinal tubules which may result in an infection of the pulp. Moreover, the bacteria and their toxins in infected root canals may go the other way, which will lead to disturbances in the healing process of periodontitis (Figure II.3).

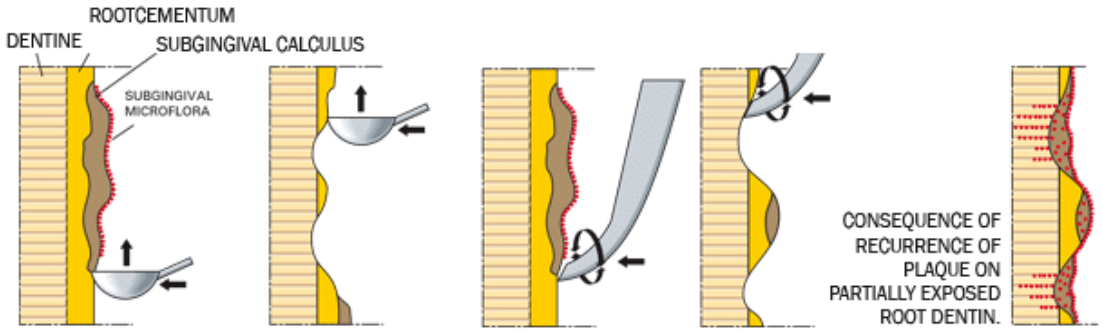


Figure II.3. Consequences of cementum over-instrumentation (Axelsson)

The assessment of the debridement of the root surfaces with the reciprocating system revealed that only a levelling of cementum protuberances occurred, and hence, only a slight loss of substance (Kocher et al., 2001).

The literature lacks in studies which compare simultaneously the invasive character of the three methods: manual, ultrasonic and reciprocating system, a fact which also reflects the incomplete traits of the available data for the practitioner.

There are also studies which were concentrated on aspects regarding the working ergonomics: working time to the reaching of the objective, the fatigability degree for the medic, the number of needed instruments and of instrument activations, the position of the practitioner for optimal access and visibility, all of these with the purpose for obtaining the least soliciting instrumental technique for an operator who has to repeat it for numerous times during his work activity.

The patient compliance is an extremely important issue in the management of the periodontal disease. The success of the therapy is closely related to the following of the therapeutic steps, to the consistency of the therapy sessions and to the attendance of the patient to these sessions. Therefore, there is a high impact of a certain therapy method to each patient. This is, certainly, a subjective aspect but also a key-element in an adequate

development, adapted to the status and the needs of the patient; the discomfort degree perceived by the patient during the sessions can interfere with the etiologic therapy and, specially, with the maintenance therapy.

The present research was, thus, justified by the lack of universal agreement regarding the existence of an optimal instrumentation technique, which could offer a smooth root surface, with a minimal loss of tooth substance, less soliciting for the practitioner, with maximal ergonomic traits and with the best patient compliance, adapted to the needs and to the abilities in our country.

Moreover, an adequate etiological and corrective periodontal therapy also comprises the treatment of carious and non-carious coronal lesions or the re-treatment and polishing of defectuous coronal reconstructions. At present, the most popular dental materials for plastic restorations are the light cured composite resins. Although the aeasthetic demands also require high polishability, few manufacturers recommend exactly how to finish and polish the surface of these materials. In cervical areas and particularly in proximal areas, it is very difficult to create a perfect contour and marginal adaptation of the restoration. Therefore, additional finishing and polishing is usually necessary for shaping and finishing the restorations. Rotary instruments like diamond burs and tungsten carbide burs have been widely used for shaping and polishing these areas while the reciprocating motion have been rarely used to activate the finishing and polishing instruments.

The surface characteristics of the dental restoration influence the aesthetic quality, the tactile sensation, the resistance to corrosion-abrasion phenomena and the adhesion of the bacterial biofilm. For composite resins the surface of the restoration is either the result of using Mylar matrices or finishing and polishing procedures when adjustments are necessary. During the last decades, a lot of finishing and polishing systems have been introduced on the market, most of them involving rotary instruments like burs, disks, rubber polishers and brushes used in one-step or multi-step procedures. The abrasives used to impregnate these instruments include diamond, silicon dioxide, silicone carbide and aluminium oxide.

Recently a new system has been introduced for finishing and polishing. Profine PDX system consists of a contra-angle handpiece using reciprocating movement with special finishing and polishing tips - Lamineers LTA (Dentatus AB, Sweden). One of the indications is the removal of restoration overhangs, shaping, finishing and polishing of restorations especially in the subgingival and proximal areas.

This research direction has been materialized by publishing the following papers:

1.Solomon SM, Stoleriu S, Tampu D, Agop Forna D, Martu Stefanache MA, Tanculescu O, Ioanid N, Martu S. E-SEM evaluation of root surface after SRP with Periotor tips. Mat Plast 2016; 53(4): 796-798.
https://revmaterialeplastice.ro/pdf/SOLOMON%204%2016.pdf
2.Solomon SM, Stoleriu S, Agop Forna D, Tampu D, Martu Stefnache MA, Urarescu IG, Martu S. The quantitative and qualitative assessment of dental substance loss as consequence of root planing by three different techniques. Mat Plast 2016; 53(2): 304-307.
http://www.revmaterialeplastice.ro/pdf/SOLOMON%20S%202%2016.pdf
3.Sorina Mihela Solomon, Daniel Tampu*, Doriana Agop Forna, Maria Alexandra Martu Stefanache, Silvia Martu, Simona Stoleriu. AFM comparative study of root surface morphology after three methods of scaling
http://www.revmaterialeplastice.ro/pdf/SOLOMON%20S%203%2016.pdf

II.I.1 The quantitative and qualitative assessment of dental substance loss as consequence of root planing by three different techniques

Aim of the study

The aim of the research was to establish the level of calculus and dental tissue loss, together with the quality of the dental root surface after the instrumentation performed by three techniques applied in the causal treatment of the periodontal disease – the Gracey curettes, the ultrasonic scaler and the reciprocating systems with Periotor inserts.

Materials and method

The research was conducted on 30 extracted teeth by periodontal pathological reasons. Tooth extraction was performed by a standardized procedure without applying extraction instruments on the root surface (extraction with pliers applied coronary), in order not to alter the state of the root surface. Prior to extraction the operator made an indentation with a fissure bur at high speeds and continuous water cooling marking the gingival margin and after extraction a second indentation was made, marking the level of epithelial attachment, these two markings delimiting the instrumentation and in vitro evaluation area.

The extracted tooth, held in pliers, was washed under running tap water and periodontal tissue residues were gently removed from the root surface with Gracey curette 5/6. Samples were decontaminated by immersion in 2.5% hypochlorite solution for 15 minutes and then stored in normal saline at room temperature in sterile 30 ml containers. The teeth were randomly distributed in three sample groups: group 1 (Gracey curettes), group 2 (ultrasonic scaler) and group 3 (reciprocating system). Prior to instrumentation, three operators were trained for one week in the Clinical Base Teaching Simulator in order to calibrate the operator for a specific method. Each method of SRP (Gracey curette method, piezoelectric ultrasonic scaling, and reciprocating systems with Periotor inserts) was performed by the same trained operator.

In the group scaled with Gracey curette (Figure II.4) each experimental surface was instrumented by applying 20 overlapping working strokes in vertical direction using a new and sharpened Gracey's curette 5/6 (Hu-Friedy Mfg. Co., Inc.) by one operator who performed an effective planing with a 60-70° working angle and applying an appropriate amount of pressure during the strokes. After instrumentation of a tooth, the Gracey curette was sharpened on an Arkansas stone.

In the group that received ultrasonic scaling, the root specimens were scaled using a periodontal tip mounted on an ultrasonic hand-piece (Satelec P5, Acteon Group, Ltd.) working at 25 kHz for 15 s (20 strokes) in a vertical direction under abundant water irrigation (Figure II.5).

A new concept of instruments, Periotor (Figure II.6), due to its design, eliminates problems adapting the instrument to complex root morphology. The instruments are mechanically driven with reciprocating strokes of 1-1.5 mm length. The instrument is placed against the subgingival plaque biofilms and calculus on the rough root cement and is designed so that when the working side faces the root surface, plaque biofilms and calculus can then be scraped off and the rough root cement planed when reciprocating the instrument in the direction of the arrows along the root.

The quantitative evaluation of hard tissue loss after performing SRP by the three methods was done by weighing each tooth before and after performing the procedure (Figure II.7). The hard tissue loss was reported for each dental unit by calculating the difference between the initial and final weight of the teeth.

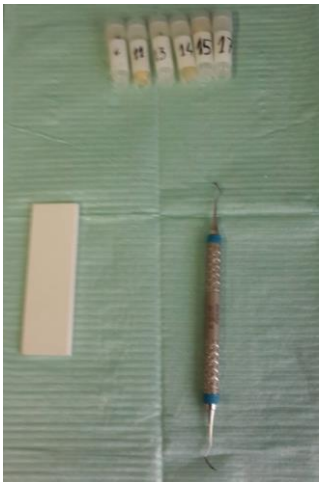


Figure II.4. The Gracey curette 5/6, together with the instrumented samples and the Arkansas stone

Figure II.5. The ultrasonic piezoelectric system, together with the instrumented samples

Figure II.6. The reciprocating system with Periotor insert, together with the instrumented samples

For the qualitative analysis of the instrumented root surfaces we applied the Roughness Loss of Tooth Substance Index (RLTSI), according to the following criteria: (0)- there is a smooth and even root surface, without marks from the instrumentation and with no loss of tooth substance; (1)- there are slightly roughened or corrugated local areas confined to the cement; (2)- there are definitely corrugated local areas where the cement may be completely removed, although most is still present; (3)- there is considerably loss of tooth substance, with instrumentation marks into the dentin. Large areas of the root are completely denuded of cement, or there are a considerable number of lesions from instrumentation.

Figure II.7. The scale used for the weight measurements before and after the instrumentation

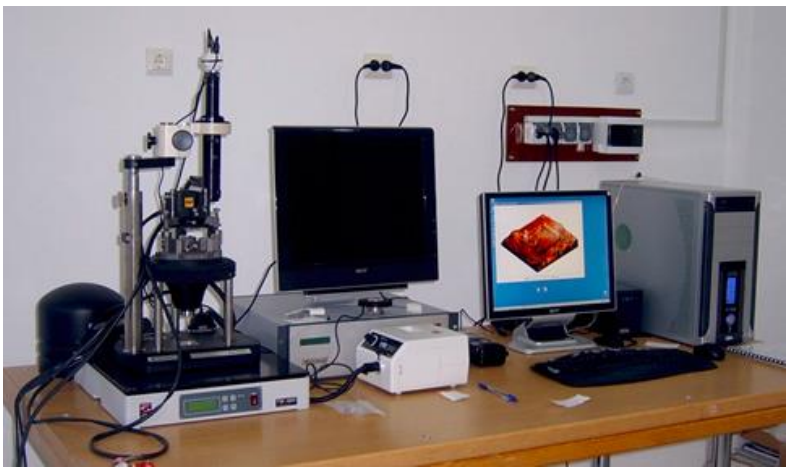


Figure II.8. AFM System

The surface morphology was investigated with a complete system AFM - Atomic Force Microscope and SPM - Scanning Probe Microscope model SOLVER PRO-M, NTMDT Russia (Figure II.8).

Atomic Force Microscopy (AFM) images were taken in air, at room temperature. The apparatus was operated in semi-contact mode, 256x256 scan point size, at different scan areas (foursquare with the side of 50, 20, 10, 5, 2, 1μm). NSG10/Au Silicon tips with a 10 nm radius of curvature and about 255 kHz oscillation mean frequency was used; the scan

velocity wax 1ÅÄ 50nm/s (depends on roughness at different scan areas). To achieve data and process them, Nova 1443 and IAP9 soft were used.

Also, a Phase Contrast Mode technique was used in order to relieve the existence of two difference phases / materials; this technique is specific for SPM and it is not possible in other instruments who investigate the nanometer architectures (SEM or TEM for example). When there are material heterogeneities on the surface, an AFM technique, namely the phase-contrast imaging method, can analyse them. In this case, simultaneously with the amplitude of the cantilever oscillation, the change in the phase of the oscillation is registered and mapped to obtain the phase-contrast image of the sample.

Before investigation, the teeth have been conditioned by ultrasound treatment in isopropylic alcohol, drying and keeping for two hours in preliminary vacuum in order to remove the dust and the monomolecular water layers from the surface.

For every tooth have been analysed three different zones on the surface and for every zone we investigated concentric foursquare areas with the size of 0.5 / 1 / 2 / 5 / 10 / 20 μm. For every area, bi-dimensional-2D and three dimensional- 3D images, phase contrast and statistical parameters have been registered.

There are two possibilities to analyse data: directly, by the images comparing and statistics. From the images comparing one can observe the differences in surface morphology, the nano-architectures and their dimension, the homogeneity or irregularities, phase separation, the roughness, the defects, etc. Statistical Analysis is another way to compare the surface morphology of scaled teeth. As opposed to inorganic material (for microelectronics, sensors, solar cells, etc.), the soft materials (biological, polymers, etc.) have a high roughness and can be different from an area to other. Methodology: every tooth (total number 30, 10 for every scaling technique: G, US, PP) was investigated in three different zones (a, b, c); on each zone, the data from 6 concentric foursquare areas with the size of 0.5 / 1 / 2 / 5 / 10 / 20 μm were registered (totally 540 foursquare). For every foursquare, the statistical parameters of roughness were calculated, totally 540 data batch. For every foursquare, the SPM instrument get vertical data for 256(on X)Å~256 (on Y) pixels = 65536 pixels. Most used roughness parameter is Root Mean Square, Sq, with the next definition:

$$Sq = [1/nÅ{\sim}(x12 + x22 + x32 + ... xn2)]1/2$$

where n is the number of investigates points (pixels). For every zone, all the averages values were calculated; then all the means for G, US, PP were computed and plotted.

Evaluation of root surface morphology following SRP using the three methods was made by quantifying the presence of Root Surface Smear Layer (RSSL). All instrumented root surfaces have been evaluated using a new method – Environmental Scanning Electron Microscopy (ESEM) which offers high advantages: the desiccation of the samples is not necessary (this step can also generate artefacts, with high risk of errors), nor is the surface coating with gold-palladium, the samples thus being available for further and repeated investigations. This ESEM method, by our knowledge, has not been previously used in the assessment of the treated dental surfaces. The micrographs were assessed using the index of Root Surface and Smear Layer Morphology: grade 1 - thick and compact smear layer, no dentin tubules open; grade 2 – thin smear layer, no presence of dentin tubules; grade 3 – residues of smear debris partially occluding dentin tubules; grade 4 - absence of smear layer on the dentin specimen.

All instrumented root surfaces were fixed on aluminium supports and the surface morphology of the uncoated samples was examined using an environmental scanning electron microscope (ESEM) type Quanta 200 (FEI), operating at 20 kV with secondary electrons in low vacuum mode (60 MPa), with a large field detector. Micrographs at four

different magnifications (Å~200, Å~1000, Å~2000 Å~5000) were recorded for each sample. The micrographs were evaluated using the Index of Root Surface and Smear Layer Morphology characteristics (IRSSLM, shortly RSSL). A single value was assigned for each sample after the evaluation of the representative images, resulting in 10 values per group and a mean value of the RSSLM was recorded in each group as a result of 10 samples values.

Data obtained from the in vitro studies was electronically stored. To determine the normality of distributions, we used the standard error of each of the two indices to calculate the confidence interval limits. If within a 95% confidence interval the value 0 was present (characteristic of a normal distribution), the distribution has a symmetry or normal flattening, which allows for comparison of parametric tests data (t test for paired samples, ANOVA single factor). If the distribution of values had been asymmetrical and with an abnormal flattening, the nonparametric tests were applied to compare data (Mann-Whitney, Wilcoxon or Kruskall-Wallis).

Results

Weighting and RLTSI results

The greatest weight loss in the samples was registered by Group 1 (Gracey curettes), with a mean value of 0.0325g. The weight loss for both Group 2 (ultrasonic scaler) and Group 3 (reciprocating system) presented almost equal values (0.0230g and 0.0232g, respectively). There were no statistically significant differences between the mean values of the weights between the study groups (Wallis and Man-Whitney tests, $p>0.05$, data not shown).

The mean values of the RLTSI score were significantly different between groups 1 and 3 ($p<0.05$) (Table II.1 and Table II.2), with better scores for the reciprocating system. The RLTSI values were statistically significantly correlated for the Gracey curettes instrumentation and for the reciprocating system with Periotor inserts. We could not find any significant correlations for the ultrasonic scaling technique.

Table II.1. The analysis results of the Kruskal Wallis Test when comparing the RLTSI values between the three groups

Group	Mean Rank	Chi-Square	Df	Asymp. Sig.
Group 1	**25.75**	12.978	2	0.002
Group 2	**18.50**			
Group 3	**11.25**			

Table II.2. The analysis of the RLTSI values between groups

		Mean Rank	Sum of Rank	Mann-Whitney U	Wilcoxon W	Z-value	Aysmp. Sig.
Group 1 vs. Group 2	Group1	15.00	180.00	42.000	120.000	-1.945	0.052
	Group 2	10.00	120.00				
Group 1 vs. Group 3	Group1	17.25	207.00	15.000	93.000	-3.625	0.000
	Group 3	7.75	93.00				
Group 2 vs. Group 3	Group 2	15.00	180.00	42.000	120.000	-1.811	0.070
	Group 3	10.00	120.00				

AFM Results

The Figure II.9 shows an example for direct images comparing, for the three teeth prepared by the three methods (G, US, PP). One can see different architectures on surfaces who can be correlated with the scaling technique used.

In samples instrumented with Gracey's curette, the nano-architecture is rounded but the dimensions of defects is medium, while in samples instrumented with US the surface morphology looks very sharp and in samples instrumented with reciprocating system PP one

can see the mildest roughness and nano-architecture.

For all samples the value of phase contrast is about 5°÷10°, that means a good homogeneity and not phase separations. At the samples PP appears a surface texture, with 67.14 degrees orientation (Figure II.10) and the hills are not symmetrical (due to the way Periotor tips mounted into Profin reciprocating handpiece operate).

The statistical parameters of surface roughness for 5 μm section of one sample when ultrasonic scaling technique was used are presented in Table II.3a. The root mean square recorded was 105.26 nm.

The calculated average values in the foursquare areas with the size of 0.5, 1, 2, 5, 10 and 20 μm are showed in Table II.3b.

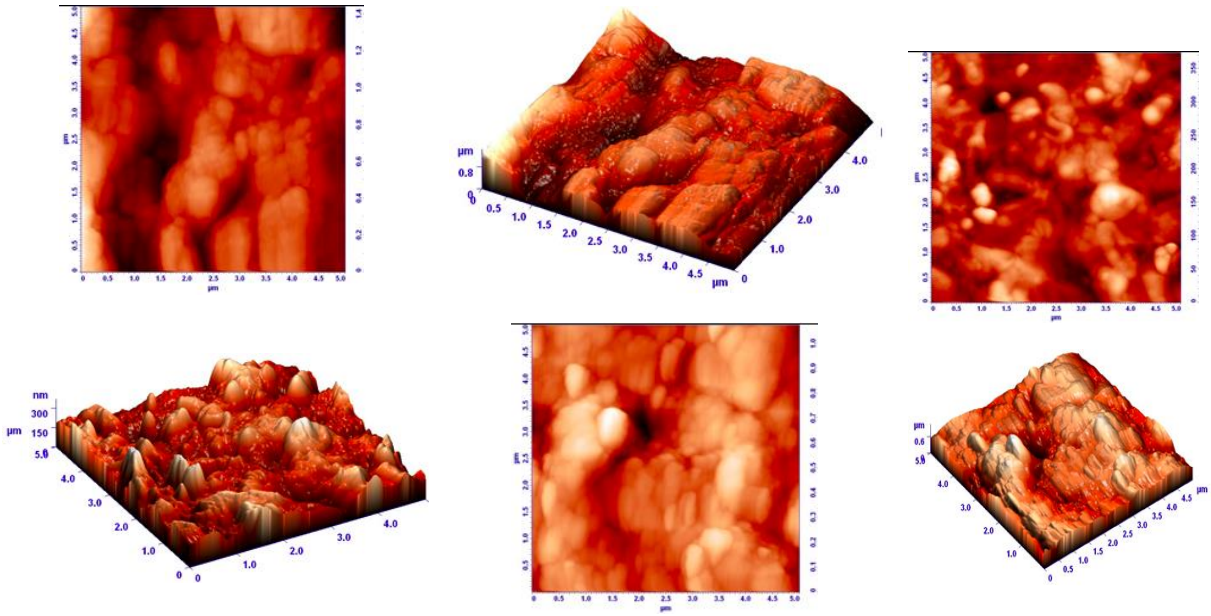


Figure II.9. Images in 2D and 3D of cementum surface instrumented with: Gracey's curette (G), ultrasonic tip (US), and Periotor tip (PP).

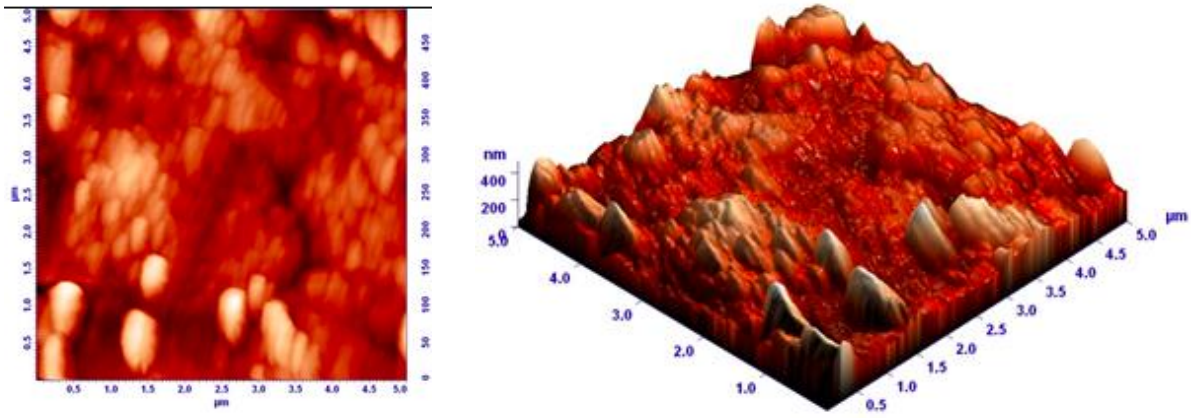


Fig. II.10. Example of 2D and 3D aspect of surface texture showing certain orientation, due to instrumentation with Periotor tips mounted in Profin counterangle handpiece (reciprocating system)

Table II.4 shows the averages for root mean squares of surface rugosity for the three considered scaling techniques for al four squares that have been analysed.

In our study Periotor device and Gracey scalers conducted to almost similar roughness of the root surface (Figure II.11).

E-SEM Results

Examples of micrographs at four magnifications (Å~200, Å~1000, Å~2000 Å~5000) registered for a sample in group 1 (sample a) and a sample in group 2 (sample b) are presented in Figure II.12.

After the examination of all micrograph at four magnifications (Å~200, Å~1000, Å~2000, Å~5000), both examiners decided to use only the micrographs at Å~200 magnification to evaluate the RSSL index due to the fact that at this magnification the entire evaluated area is visible.

Examples of the samples micrographs evaluated with grade 1, 2, 3, and 4 according to RSSL index are presented in Figure II.13 (a, b, c and, respectively, d). No large deposits of calculus were seen in all groups. Few calculus remnants were present in few samples of group 1 and 2. The presence of smear layer was noted in all three groups, more often observed in group 1. The ESEM evaluation of the samples also indicated that the surface of the samples of group 3 were smoother than those in group 1 and 2. An example of a sample in group 2 presented distinct scratches as a result of scaling instrument action is presented in Figure II.14. No irregular surface consisted in depression and elevations were present in group 3.

The total number of the score and the mean values of RSSL recorded for each group are presented in Table II.5.

Table II.3. Statistical parameters of roughness and the calculated average values per zone for one sample - ultrasonic scaling technique

Table II.3a Statistical parameters of roughness

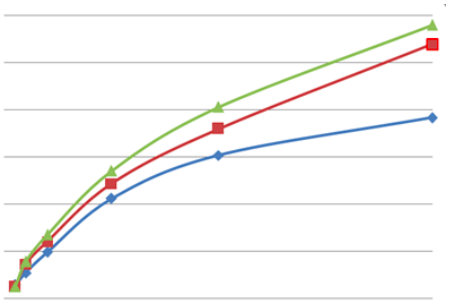
US-5_a_5µm	
Amount of sampling	65536
Max	795.253 nm
Min	0 nm
Peak-to-peak, Sy	795.253 nm
Ten point height, Sz	399.756 nm
Average	453,.392 nm
Average Roughness, Sa	79,0924 nm
Second moment	465.45
Root Mean Square, Sq	105.26 nm
Surface skewness, Ssk	-0.129816
Coefficient of kurtosis, Ska	0.769674
Entropy	12.5921
Redundance	-0.30712

Table II.3b. The calculated average values per zone for one sample number 16 – ultrasonic scaling technique

	Root Mean Square, Sq (nm)			
l (µm)				
	US-16_a	US-16_b	US-16_c	media
0.5	7=.23921	7.39341	10.6479	8.43
1	14.67	11.95	22.4846	16.37
2	36.7635	25.6608	29.8687	30.76
5	67.3703	52.0857	55.7551	58.40
10	72.2807	72.2358	77.6623	74.06
20	88.7042	114.261	132.082	111.68

	Root Mean Square, Sq(nm)		
l (µm)	US sr1	PP sr2	G sr3
0.5	14.95	12.43	12.78
1	27.50	36.77	39.45
2	49.41	60.70	67.71
5	106.12	122.00	135.71
10	152.09	180.32	203.04
20	191.92	269.93	290.32

Table II.4. The average root mean square of surface rugosity for all analysed foursquares



G = green (1)
PP = red (2)

US = blue (3)
(vertical: Sq / orizontal:1µm)

Figure II.11. Distribution curve of the means average values for the three scaling techniques: G, US, PP;

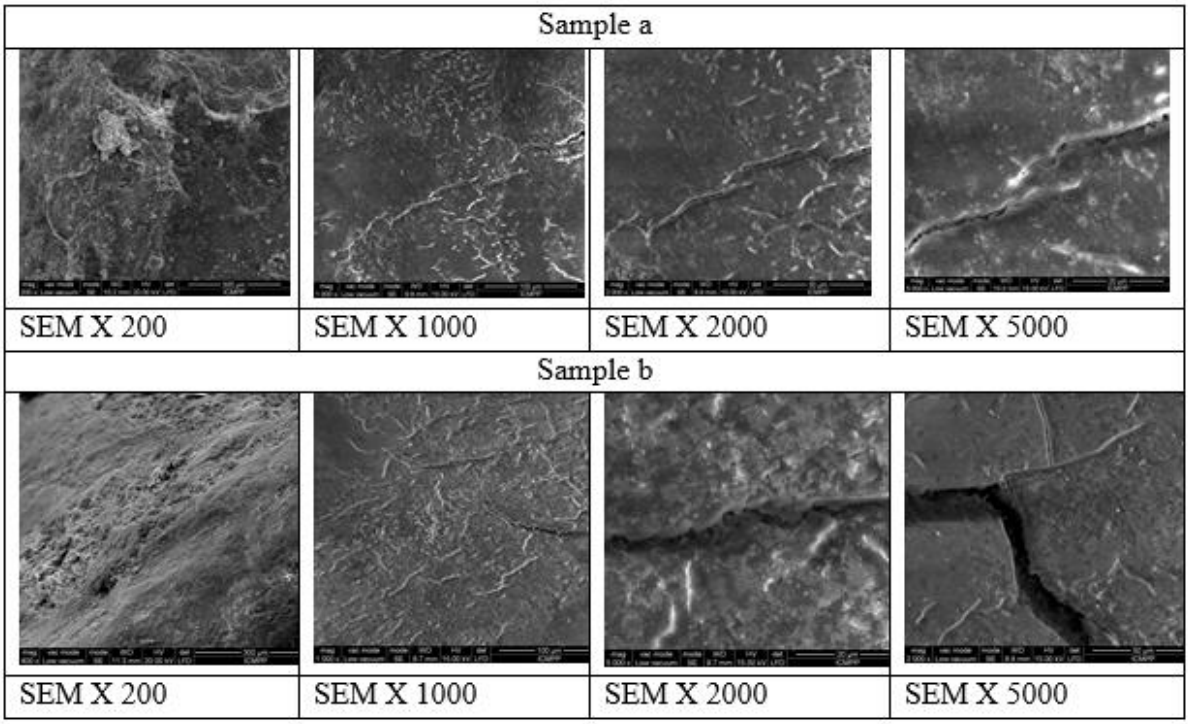


Figure II.12. Micrographs at four magnifications registered for a sample in group 1

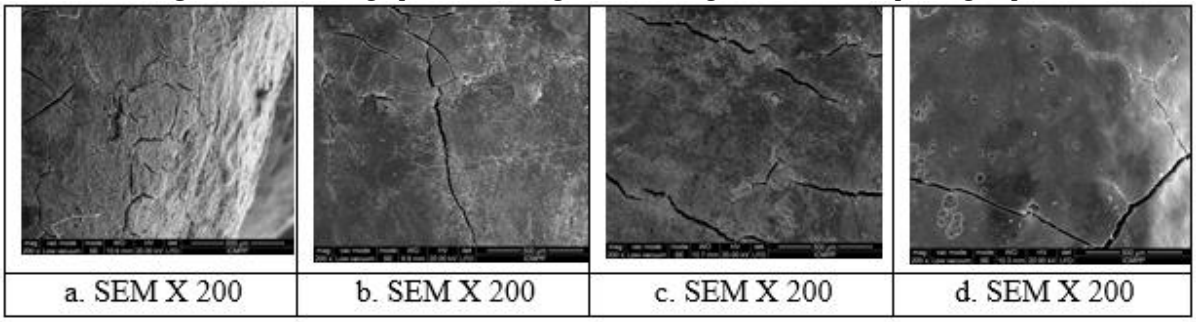


Figure II.13. Samples micrographs evaluated with grade 1, 2, 3, and 4 according to RSSL index

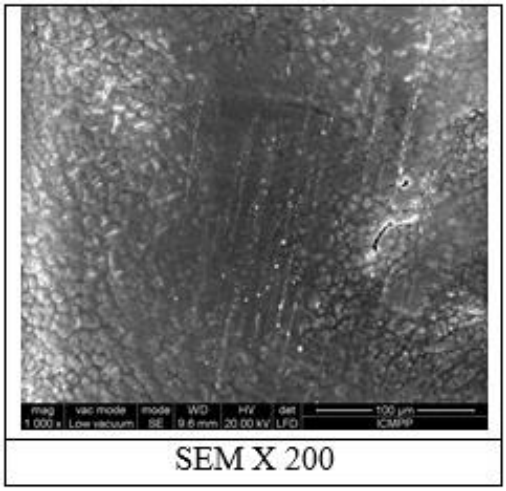


Figure II.14. Micrograph of a sample in group 2 presented distinct scratches

Table II.5. Total number of the score and the mean values of RSSL index

Groups	No.of samples	Total no. of scores				Mean RSSL
		1	2	3	4	
Group 1	10	5	3	2	-	1.7
Group 2	10	1	6	3	-	2.2
Group 3	10	-	4	6	-	2.6

Discussion

In our study the morphological aspect of root surface when using hand curette revealed a compact smear layer in many samples. The same results were also obtained in other studies (Aspriello et al., 2011). Hand instruments often produce irregular pattern of root morphology, especially when combined horizontal and vertical strokes were applied. More than that, one of our previous study demonstrated that the use of Gracey curettes was the most aggressive method for SRP, leaded to the highest amount of dental hard tissues lost (Solomon et al., 2016b).

In AFM measurement, the roughness increases with the dimension of investigated area (not linear), especially at soft materials; this situation appears because on extended areas increases the probability to have defects and nanostructures with large dimensions. In figure 2, the differences in surface morphology appears very clear.

In many studies determination of the surface roughness was performed using profilometer device. In the present study, AFM evaluation was used, which is a modern and precise method. AFM investigation can give information related to the surface morphology of dental hard tissues by assessing their roughness (Stoleriu et al., 2012). The mandatory flat surface of the samples in profilometric measurements was no longer needed.

A lack of common opinion regarding the effect of different type of instrumentation on root surface is still in the literature. Some researches pointed that manual instrumentation might lead to massive root surface removal, while other researchers reported same effects when using ultrasonic scalers (Oda et al., 2004).

When the root surface texture was evaluated in previous studies, it was showed that rotating instruments leaded to numerous scratches, while Gracey scalers determined a different texture of the surface, with a more important roughness. However, the surface roughness measurements revealed no differences between the tested instruments (Kishida et al., 2004). These controversial results might be explained by the shorter distance in

evaluation the surface roughness using the profilometer.

In our study Periotor device and Gracey scalers conducted to almost similar roughness of the root surface. The results of our research are in contradiction with other studies which have showed that the hand instruments produced smoother surface when compared to ultrasonic instrumentation (Kocher et al., 2001). This can be explained by the fact that the tip of ultrasonic instruments is thinner than the cutting edge of the hand instruments, in this way causing lesser damages on root surface. In our study the root morphology of many samples after curettes action presented distinct and even large area of dentine tubules opening as a result of an aggressive action of the instrument. Even a hand instrument delicately used have the potential to induce scratches and irregularities on the root surface due to the microscopic roughness of the cutting edge.

In this study no distinct tracks of instrument action were observed, probably due to calibrated pressure and to only vertical strokes applied by the operator. Ultrasonic instrumentation created only small irregularities characterized by several pits and partially covered by a thin and porous layer of debris.

Also, our other study concluded that ultrasonic technique determined the smoothest root surface when comparing the same three methods for SRP according to the surface morphology evaluated using AFM (Solomon et al., 2016a).

In another study (Iovan et al., 2016) we investigated a particular type of reciprocating system – Profine, for restaurations finishing and polishing with the diamond and carbide Lamineer tips. Significant differences of the surface roughness have been found between some of the tested materials, for both types of Lamineer tips. The surface roughness of composites seems to be dependent on both material and type of Lamineer used for polishing.

Although the Lamineer system have shown promising result for finishing composite restorations, polishing seems to be necessary in order to reduce the surface roughness of the tested materials beyond the threshold value of 0.2μ.

The smoothest surfaces were created by using Mylar matrices for all the tested composite shades. Using reciprocating movement with special diamond and carbide tips seems to be a promising technique for shaping and finishing composite restorations in areas difficult to be reached. However, the average values of surface roughness for the tested materials have been higher than threshold value of 0.2 μ, excepting the Enamel shade finished with diamond Lamineer tips. Therefore, additional polishing procedures should be used in order to improve the final gloss of the surface. Further evaluation of the impact of several factors like polishing time and variables related to operator is necessary.

The surface roughness of the tested composite material depended on the composite shade and the type of Lamineer used for finishing procedures. For the enamel shade the surface roughness was lower when using the diamond tips, while for dentin shade and universal shade the surface polishability was higher when carbide tips had been used.

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The surface roughness of the tested composite material depended on the composite shade and the type of Lamineer used for finishing procedures. For the enamel shade the surface roughness was lower when using the diamond tips, while for dentin shade and universal shade the surface polishability was higher when carbide tips had been used.

Conclusions

The results of our in vitro research, conducted by instrumentation of the extracted teeth root surfaces by three methods – manual Gracey curettes, piezoelectric ultrasonic scaler and reciprocating system with Periotor inserts – revealed the fact that the scaling in deep pockets with the Periotor inserts was the least aggressive method, followed by the ultrasonic scaler and the Gracey curettes. Surface morphology obtained after instrumentation with Periotor inserts is similar to that obtained by instrumentation with Gracey curette, and caused a "deletion" of the relief in the direction of instruments action. Our AFM study of the root surface morphology consecutive three scaling techniques put in first place the ultrasonic technique, which determined surfaces with the lowest average of root means square.

Our study showed that according to the root surface morphology evaluated using the RSSL index on micrographs obtained by environmental scanning electron microscopy (ESEM), the use of Periotor inserts mounted in Profin handpiece created smooth surfaces without organic debris and also without wide denudation of dentine. Almost similar root surface morphology was obtained using the ultrasonic scaling technique with special perio-tips and operating mode specially tuned for access to deep periodontal pockets up to 10 mm deep. The less quality of root surface morphology was obtained by using the hand scaling technique with Gracey curette, which leaded to extensive areas of dentinal tubules denudation and scratches.

II.2 Effects of non-surgical conventional periodontal therapy in systemically impaired patients

The literature is abundant data regarding the potential beneficial effect of scaling and root planing even on general level in patients with different forms of systemic pathologies; nevertheless, the data is inconsistent and there is not a universal consensus regarding the protocols for such patients. Therefore, in my research an important focus was on the effects of SRP on systemically impaired patient.

This research direction materialized by publishing the following papers:

1.Solomon S, Pasarin L, Ursarescu I, Martu I, Bogdan M, Nicolaiciuc O, Ioanid N, Martu S. The effect of non-surgical therapy on C reactive protein and IL-6 serum levels in patients with periodontal disease and atherosclerosis. Int J Clin Exp Med 2016; 9(2): 4411-4417.
http://ijcem.com/files/ijcem0015062.pdf

2.Sincar CD, Ioanid N, Rudnic I, Martu I, Solomon SM, Pavel LL, Rezus C, Martu S, Plesea Condratovici C. The biochemical effects of non-surgical periodontal therapy in patients with and without chronic renal disease. Rev. Chim (Bucharest) 2017; 68(3): 605-607.
http://www.revistadechimie.ro/pdf/39%20SINCAR%20C%20D%203%2017.pdf

3.Ursarescu IG, Martu Stefanache MA, Solomon SM, Pasarin L, Boatca RM, Caruntu ID, Martu S. The assessement of IL-6 and Rankl in the association between chronic periodontitis and osteoporosis. Rev. Chim (Bucharest) 2016; 67(2): 386-389**.**
http://www.revistadechimie.ro/pdf/URSARESCU%20I%202%2016.pdf

II.2.1 Impact assessment of non-surgical periodontal therapy in patients with cardiovascular diseases

Aim of the study

More and more epidemiology studies confirm the association between periodontal disease and atherosclerotic coronary disease. These two diseases (chronic periodontitis and atherosclerosis) have complex ethology, genetic predisposition and many common risk factors, smoking being the most significant one. Chronic periodontal infection may contribute to atherogenesis process, to the evolution of this process on arterial levels. Periodontal disease, as cardiovascular disease, has inflammation as a common aspect. Local periodontal inflammation stimulates the systemic inflammation. We conducted a study with the purpose to establish if the resolution of the periodontal infection can have an impact on the serum markers of the systemic inflammation in patients with risk of atherosclerosis and to assess the systemic effects of the treatment by examination of the changes determined by the treatment on the systemic markers of inflammation and on the cholesterol levels.

Materials and method

This prospective study was conducted on a number of 64 subjects with generalized advanced PD. The inclusion criteria for the patients were: presence of generalized advanced periodontitis, without any other signs of infection, periodontal pocket depths higher than 6 mm and alveolar bone resorption higher than 30% on at least 50% from the present teeth. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

The exclusion criteria were: history and/or presence of infectious diseases of any type, antibiotic treatment in the last 3 months, treatment with any drugs which can influence the serum levels of inflammatory markers, periodontal treatment in the last 12 months, pregnancy or lactation, any other inflammatory disease which could alter the periodontal status and the systemic results.

The serum and periodontal parameters were assessed baseline and at 3 months after the completion of non-surgical periodontal treatment. At each examination we conducted: periodontal probing, recession examination (from the free gingival margin to the enamel-cementum junction), bleeding on probing assessment in 6 sites for each tooth, bacterial plaque index evaluation, expressed in percentages of surfaces entirely covered (O'Leary). The patients were submitted to non-surgical periodontal treatment and were informed regarding the oral hygiene methods. We performed scaling (manual and ultrasonic) and root planning with Gracey curettes under local anaesthesia. The therapy was not limited by time and number of therapy sessions, being completed after 1-3 months from the first session.

Venous blood was collected from the cubital vein baseline and at 3 months after the treatment. The serum was obtained by centrifugation at 200rpm for 15 minutes. The samples were maintained at -70^{o}C before the analysis and were submitted to a standard interpretation, to avoid inter-individual variations.

The CRP serum levels were determined by immunoturbidimetry with a minimum detection limit of 0,25mg/l (Cobas Integra 700, Roche, Mannheim, Germany). The IL-6 serum levels were determined by enzyme-linked immunosorbent assay (ELISA) (HS600 Quantikine, R&DInc., Minneapolis, USA, detection limit of 0.04 ng/l). The levels of total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides were determined by standard laboratory tests.

All the obtained data were registered and statistically analysed. Normally distributed variables are reported as mean ± standard deviation; with 95% confidence intervals (95% CI) (the results are expressed with one standard deviation). Changes in serum concentrations of CRP were tested by one-way analysis of covariance as primary outcomes. Changes in IL-6,

triglycerides and total/LDL/HDL cholesterol were similarly tested as secondary outcomes. Age, gender, body mass index and cigarette smoking were subsequently included as covariates. For the data analysis we used the PASW 18 Statistics software. The laboratory results were reported to clinical changes of periodontal probing depths and gingival recessions. Significance was set at $p < 0.05$.

Results

From 79 patients, only 64 subjects agreed to the treatment plan and followed it until the end-point of the study. The mean age was 46±7 years. The demographic data are presented in Table II.6.

Table II.6. The demographic data of the included subjects

Age (years)	46± 7	
Gender	Female 36 (56.25%)	Male 28 (43.75%)
Residence	Urban 43 (67.18%)	Rural 21 (32.82%)
Smoking status	Yes 41 (64.06%)	No 23 (35.94%)
BMI (kg/m^2)	27.5 ± 2.3	
Family history of CVD	Yes 27 (42.18%)	No 37 (57.82%)
Periodontal diagnosis	Chronic periodontitis 61 (95.31%)	Aggressive periodontitis 3 (4.69%)
BMI: Body mass index; CVD: cardio-vascular diseases		

At 3 months after treatment, we observed that the oral hygiene had significantly improved as can be observed in Table II.7.

The mean serum value of CRP at baseline was 1.9 ±1.1 mg/l, with no differences regarding the age, gender, periodontal diagnosis or smoking. The CRP values were associated to BMI and mean bone loss. The mean value of CRP at 3 months after treatment was reduced (p<0,001) to 1.5 ±0.9mg/l (Table II.8). The serum levels of IL-6 also presented a significant reduction, from 1.2ng/l to 0.8ng/l (p=0.006). Lipid profile showed no major variation.

Also, no significant changes in medication and smoking habits after 3 months were reported.

Table II.7. Periodontal parameters (baseline and at 3 months after treatment)

Periodontal parameter	**Baseline**	**At 3 months**
	Mean Value (± SD)	Mean Value (± SD)
Plaque Index	62.05 ± 19.54	23.80 ± 12.34
BOP sites	64.47 ± 15.36	17.41 ± 12.37
Periodontal pockets >4mm (mm)	78.23 ± 12.33	31.42 ± 13.37
Probing depth	4.59 ± 0.48	3.15 ± 0.27
Recession (mm)	2.56 ± 0.47	2.55 ± 0.74
CAL (mm)	5.03 ± 2.43	3.76 ± 1.54

Table II.8. Values for CRP, IL-6, total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides at baseline and after 3 months

Parameter	Baseline mean value (±SD)	Mean value (±SD) after treatment
CRP (mg/l)	1.9 ± 1.1	1.5 ± 0.9
IL-6 (ng/l)	1.2	0.8
Total cholesterol (mmol/l)	5.4 ± 0.7	5.3 ± 0.7
LDL cholesterol(mmol/l)	3.3 ± 0.6	3.1 ± 0.6
HDL cholesterol (mmol/l)	1.4 ± 0.5	1.3 ± 0.4
Triglycerides (mmol/l)	1.3 ± 1.1	1.2 ± 0.8

The periodontal parameters reveal the severity and the extent of the periodontal infection. The values of the periodontal parameters (BOP, probing depth, CAL) significantly improved after the periodontal treatment. This aspect can be related also to the improvement of the oral hygiene. This finding was also observed in a previous study on CVD patients who underwent scaling and root planing (Pasarin et al., 2011); after SRP was performed, oral hygiene was significantly improved, plaque index average 20% between 2 and 6 months. Bleeding indices decreased and reached average values of 16% at 2 months and 17% at 6 months (initial value was 63.57%).

Subjects presented a decrease in number of periodontal pockets from 77 23 at first meeting to 28 16 at 2 months and 23 15 at 6 months. Mean periodontal pocket depths decreased from 4.36 mm at baseline, to 3.25 mm at two months and 3.19 mm at 6 months. Gingival recessions have stagnated and attachment loss mean values improved from 4.93 mm at baseline to 4.74 at 2 and 4.85 mm at 6 months.

Discussion

The serum levels of CRP at baseline were at the superior level of normal values, most probably associated with an acute infection or a systemic inflammatory impairment. This results must be taken into consideration: the specialist has to evaluate the serum levels of CRP in the context of an infectious disease, as an indicator of systemic inflammation and of therapy possibilities.

Our results suggest a possible role of untreated severe periodontitis on potential atherosclerotic processes via systemic inflammation. The subjects with an improved periodontal status after 3 months, with correction of other co-factors (age, gender, periodontal diagnosis), also presented low levels of CRP and IL-6. These observations demonstrate the relationship between the therapy and systemic parameters. A randomized trial in patients with refractory hypertension found that periodontal therapy resulted in significant reduction of CRP (35%) and fibrinogen (14.5%) in the test group (n = 11) compared with the control group (n = 11) after 3 months (Vidal et al., 2009).

Moreover, an incomplete control of the periodontal disease (persistency of periodontal pockets and BOP) is associated with high levels of CRP and IL-6. These observations demonstrate new perspectives for further studies regarding the therapy approach, preventing the systemic inflammation and ATS. The degree of improvement of CRP and IL-6 values is noticeable. The decrease in CRP and IL-6 values is comparable to the one after anti-inflammatory drugs intake. Data from other longitudinal studies show that CRP values are related to atherogenesis and other cardio-vascular events (Pasarin et al., 2011). There is a major interest regarding also the chronic infection and its association with the inflammatory response.

Biologically, smoking can exert an adverse effect on the fibroblasts function, chemotaxis and phagocytosis of neutrophils, immunoglobulins production and peripheral vasoconstriction. In this study the number of smokers was high (64.06%). The immune response is impaired in smokers, this fact being related to the affected functions. Therefore, smoking and systemic and local infections can determine endothelial irritation and/or pro-inflammatory cytokines stimulation, affecting the immune response of the host, with tissue lesions. Smoking presents possible pathogenic properties in PD and ATS, being recognized as a risk factor (Naji et al., 2019).

In our study we did not observe important changes in the lipid profile. Bacterial lipopolysaccharides can significantly influence the lipid profile, determining also changes in the insulin secretion (Blasco-Baque et al., 2017). These aspects require supplementary research. The present study may present certain limitations because we did not assess the

global level of other systemic inflammatory markers (such as IL-1 and TNFα) and the genetic susceptibility of the host.

Following the periodontal non-surgical therapy (scaling, root planning), the periodontal parameters presented a noticeable improvement (reduced bleeding on probing, clinical attachment loss and probing depths); the diminished clinical signs of inflammation and tissue loss are the clear localized image of an improved periodontal status.

Furthermore, after the periodontal treatment values of serum CRP and IL-6 decreased significantly. This result suggests that an improved local periodontal status can reduce the risks and effects associated with inflammation, like progression of ATS and its complications.

Changes of the lipid profile between baseline and the 3-month follow-up were minor. This fact could be related to the lack of diet changes or to the bacterial metabolism, further researches being necessary.

Conclusions

The main conclusion of our study is that interdisciplinary protocols can emerge to refine management of ATS patients like screening for PD, adjustment of medication doses to reduce side effects and more, which are subject of further research. This more complex approach of the atherosclerotic patient could lower the risks and complication of the disease and reduce side-effects of medication. These in turn could improve survival and quality of life in these patients and also ease the burden of spending on medication.

II.2.2 The biochemical effects of non-surgical periodontal therapy in patients with and without chronic renal disease

Aim of the study

Studies have shown an association between high levels of biomarkers and periodontitis, association which decreases after the periodontal treatment. Because of this association with systemic inflammatory response, chronic periodontitis was included as a risk factor for chronic renal disease. The fundamental idea of the study is based on the fact that the main purpose of patients with chronic renal disease management at present and in the future is to ensure a normal life full and independent. Recovery integration means, specific of medicine, according to results of the studied analysis parameters, will ensure the success of multidisciplinary therapy approach to the management of these patients.

Specifically, in this study we hypothesized that a part of chronic inflammatory response observed in patients with chronic renal disease is due to physiopathological reactions caused by the presence of chronic periodontitis, which, during the course of its evolution, induces an increase in the expression of inflammatory markers.

Materials and method

This study was conducted in the Specialty Ambulatory of Emergency Hospital St. Andrew Galati, Department of Nephrology. Also the study was done in collaboration with the Department of Periodontology of the Faculty of Dental Medicine of the University Gr. T. Popa, Iasi.

Evaluation of oral health in patients undergoing dialysis occurred in the hospital (for non-transportable patients), in the Department of Periodontology and in own private practice from Galati. All patients who had a diagnosis of chronic periodontitis received periodontal treatment. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

Patients were divided into two groups: the first group consisted of patients with chronic renal disease and periodontal disease who were undergoing periodontal treatment (test group)

and the second group, control group, composed of patients without any systemic disease, but who experienced moderate to severe chronic periodontitis, also periodontal treated.

This study included patients over 18 years' old who have not received in the last 6 months any periodontal, antimicrobial or anti-inflammatory treatment, and have not used steroids or immunosuppressive drugs.

Blood samples were taken for biochemical analysis at baseline and at 3 months after periodontal therapy. Venous blood was collected in vacuum tubes between 7:00 am and 9:00 am after 12 h after the last meal. A tube containing EDTA was analysed for blood following parameters: albumin, uric acid, creatinine, urea.

For comparisons between the group with chronic renal disease and the control group was used student t-test for independent samples or Mann-Whitney nonparametric test. For comparison before and after the periodontal therapy it have used the t-test or Wilcoxon signed rank test. Analyses were performed using SPSS 13.0 computer program V.

Results

Patients in the study groups were homogeneous demographic characteristics and periodontal therapy was the only variable in both groups. It is important to emphasize that no patient did not use statins or iron replacement therapy during the study. The study was conducted from March 2013 to August 2014 and was completed after participants monitoring was complete. It is noted that the percentage of men is 63% and is significantly higher than women in the group with chronic renal disease. In the control group the percentage of men (55%) was lower than in the group without chronic renal disease but still predominant.

For serum albumin, the average value before periodontal treatment is 4.90g / dL for the group of patients without renal disease and 3.70g / dL for the group of patients with renal disease. The average values after periodontal treatment, are relatively enlarged and inverted for groups of patients studied, measuring 5.40g / dL for the group of patients without renal disease and 4.15g / dL for the group of patients with renal disease.

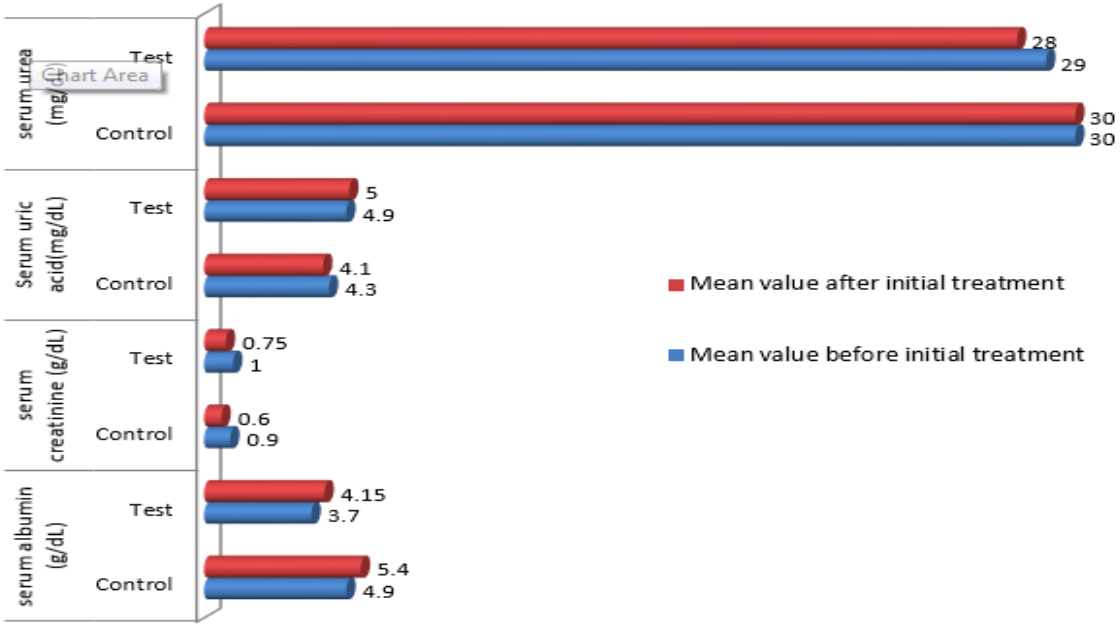

Figure II.15. Serum markers - before and after initial periodontal treatment

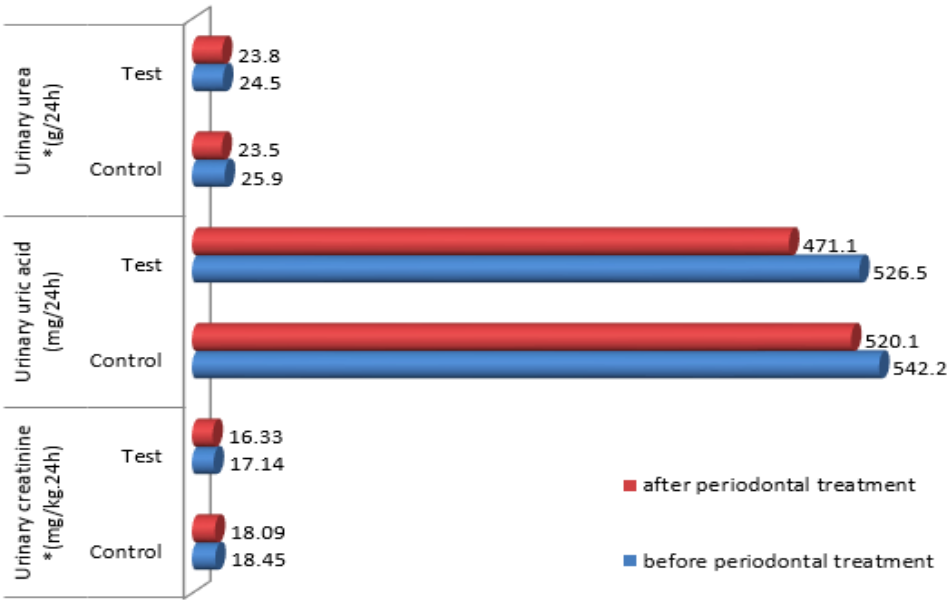

Figure II.16. Urinary markers – before and after initial periodontal treatment

In the case of serum creatinine, the mean periodontal treatment before and after the show is respectively 0.60 g/ dL for the group of patients without renal disease and 0.75 g / dL for the group of patients with renal disease.

Mean before periodontal treatment, are relatively increased for groups of patients studied, measuring 0.90 g / dL for the group of patients without renal disease and 1.00 g / dL for the group of patients with renal disease (Figure II.15).

For urinary uric acid, the mean values before periodontal treatment are respectively 542.2mg / dL for the group of patients without renal disease and 526.5 mg / dL for the group of patients with renal disease. Values are relatively reduced after periodontal treatment for groups of patients studied, with values of 520.1 mg / dL for the group of patients without renal disease and 471.1 mg / dL for the group of patients with renal disease.

For the urinary urea, the average value before periodontal treatment is 25.9 g / 24h for the group of patients without renal disease (control) and 24.5 g / 24h for the group of patients with renal disease. The average values after periodontal treatment, are 23.5 g / 24h for the group of patients without renal disease and 23.8 g / 24h for the group of patients with renal disease (Figure II.16).

Discussion

This study evaluated the impact of periodontal therapy on biochemical markers and led for the first time a causal association between periodontal disease activity and their level. We included 56 patients with chronic periodontitis, 36 with chronic renal disease and 20 without systemic disease and with normal renal function (control group).

Markers were evaluated before and 3 months after periodontal treatment. The effectiveness of periodontal treatment was confirmed by biochemical parameters improvement.

An association between periodontal disease and renal disease is often found in studies using a population where the renal disease is already diagnosed. In these cases, duration of renal end stage and type of topical and systemic treatment administered to patients significantly affect the association (Bayraktar et al., 2008). Therefore, we have shown that periodontitis may promote any detectable changes in renal function.

Thus, by analogy, in this study the test groups and control groups were compared not only with each other but also comparative analyses were performed based on the reference

values of markers of renal dysfunction.

We think it could be plausible existence of a causal link between periodontal disease and chronic renal disease both by glomerular invasion by periodontal pathogens, directly and indirectly through systemic inflammatory effect caused by chronic periodontitis.

Conclusions

Success of periodontal therapy reduce systemic inflammatory response and decreases levels of biochemical markers indicating that this may be an important intervention therapy in patients with chronic renal disease. Considering that chronic inflammation is a risk factor for atherosclerotic diseases, cardiovascular patients with hypertension and diabetes, leading causes of chronic renal disease, it is plausible that immediate diagnosis of periodontal disease, followed by periodontal therapy should be an important preventive measure in chronic renal disease in daily clinical practice.

II.2.3 The effects of non-surgical mechanical therapy on patients with periodontal disease and osteoporosis

Aim of the study

We proposed a detailed investigation of the interrelations between a local inflammatory status generated by the periodontal disease and the systemic status affected by the presence of osteoporosis.

Our objectives were to investigate the differences of IL-6 and RANKL in GCF in patients with chronic periodontitis, with and without chronic systemic disease (osteoporosis). The correlations were made to the clinic parameters of the periodontal disease.

Materials and method

The study group included 38 patients, evaluated by inter-disciplinary collaboration between the Periodontology Clinic of the Faculty of Dental Medicine of ”Grigore T. Popa” UMPh, Iași and the Endocrinology Clinic of the Emergency County Clinical Hospital ”St.Spiridon”, Iași. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

The cases were diagnosed by the T Score provided by osteodensitometry.

The periodontal examination involved inspection and palpation. The following variables were assessed:

- The Plaque Index (PI) – registered in four sites for each tooth (mesial-vestibular, center-vestibular, distal-vestibular and center-oral)
- The Probing Depth (PD) and the clinical attachment loss (CAL)
- The Bleeding on Probing index (BOP)

The initial step of the study was focused on the analysis of the Il-6 and RANKL in gingival crevicular fluid (GCF). The measurements were conducted according to the producer standards and the samples were measured in duplicate. The minimal detectable concentrations were of 0.1 pg/ml for IL-6 and 0.4 pg/ml for RANKL. The samples with concentrations under the detection limit were marked as 0.

For the statistical analysis we used SPSS 20.0 software (IBM, Armonk, NY, USA), with $p<0.05$ for significant difference. The continuous variables with normal distribution are expressed as mean ± Standard Deviation and were analysed with parametric tests (paired T Test). Due to the fact that the cytokine levels were not normally distributed, the data are expressed as minimum and maximum and non-parametric tests were used (Mann Whitney U test and Wilcoxon test). Fisher and McNemar were used to compare the frequencies between samples. The correlations between clinical parameters and cytokines were determined by Spearmen test.

Results

We examined 38 patients with chronic periodontitis, divided in two groups: the study group – patients with osteoporosis (n=20) and control group – systemic healthy patients (n=18). The mean age and the age interval for the study and control group was 55 years old (49-65 years old) and 56 years old (44-67 years old), respectively (Table II.9). We observed a higher number of urban patients for both groups. The probing depth (PD), the bleeding on probing (BOP) and clinical attachment loss (CAL) were significantly higher for the study group when compared to the controls ($p<0.05$) (Table II.10). We could not observe significant differences in plaque index between groups.

Table II.9. Demographic data for the study groups

Parameter		**Study group (n=20)**	**Control group (n=18)**
Mean age (interval)		55 years old (49-65 years old)	56 years old (44-67 years old)
Provenience	**Urban**	15	16
	Rural	5	2

The same results were obtained in another study, where we observed that probing depth (PD), bleeding on probing (BOP) and clinical attachment loss (CAL) values were significantly higher in the osteoporosis group than in the systemically healthy group (Ursarescu et al., 2014).

Table II.10. Clinical parameters for the study groups

Parameter	**Study group (n=20)**	**Control group (n=18)**
PD (mm)	4.9±0.1	3.4±0.2
CAL (mm)	1.9±0.2	0.9±0.1
BOP (%)	59.6	40.4
BOP sites (n)	31	21
PI (%)	76±2	74±1

The values are expressed as mean ± Standard Deviation (SD)
PD: probing depth; CAL: clinical attachment loss; BOP: bleeding on probing; PI: plaque index

IL-6 was the most frequent cytokine in GCF and was found in all study sites (Table II.11). We observed significantly higher values for IL-6 and RANKL in osteoporosis patients ($p<0.05$).

Table II.11. Cytokine levels in GCF samples

Cytokine(pg/site)	**Study group (osteoporosis)**	**Control group**	**p-value**
IL-6	72,03 (2,17-2099,89)	29,70 (0,75-541,58)	0.001
RANKL	0,57 (0,00-126,95)	0,09 (0,00-35,15)	0.007

GCF=gingival crevicular fluid; $p<0.05$ – statistical signification. The results are expressed as mean (minimum, maximum).

Table II.12. Correlation analysis between clinical parameters (mm) and cytokine levels (pg/site) in GCF

Correlation PD	**Q**	**p-value**	**Correlation CAL**	**Q**	**p-value**
IL-6	0.386	0.002	IL-6	0.390	0.002
RANKL	0.437	<0.001	RANKL	0.439	<0.001

GCF= gingival crevicular fluid; Q= Spearman coefficient; $p<0.05$ - statistical signification; CAL= clinical attachment loss; PD= probing depth.

In order to establish a probable clinical relevance of these observations, the correlation between clinical parameters and total cytokine levels was conducted in study sites. As shown in Table II.12, we observed a positive correlation between IL-6 and RANKL levels and PD and CAL.

Discussion

Maintaining the balance of proinflammatory and anti-inflammatory cytokines in the body is one of the manifestations of self-regulation (Garlet et al., 2004). During the past decade, considerable evidence suggests that oestrogen prevents bone loss by blocking the production of proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-10, tumor necrosis factor- (TNF-) α in bone marrow and bone cells.

Cytokines are soluble proteins which can initiate, mediate, and control immune and inflammatory responses. It has been proposed that pro- and anti-inflammatory cytokines contribute to various bone metabolic diseases including periodontitis and postmenopausal osteoporosis (Yang and Yang, 2019). Among the proinflammatory, TNF-α has been reported to present fundamental role in periodontal bone destruction.

Elevated proinflammatory cytokines in the periodontal microenvironment increase the number of osteoclasts by promoting osteoclast precursors to differentiate into osteoclasts and extending the lifespan of osteoclasts. Estrogen blocks bone loss by blocking the production of proinflammatory cytokines in the bone marrow, bone cells, and periodontal ligaments. IL-1β and TNF-α are potent promoters of bone resorption and inhibitors of bone formation, and IL-6 promotes the differentiation of osteoclast precursors into osteoclast and MMP production (Fawaz et al., 2017).

In a previous study (Ursarescu et al., 2014) we observed that TNFα clearly showed great differences between the osteoporosis group and the control group. It may be suggested that elevated GCF and serum TNFα contributes to high number of B cells and T cells present in the inflammatory periodontal tissues, enhancing the periodontal tissue breakdown. TNF-α is among the upstream cytokines that are key factors that induce the production and secretion of downstream cytokines, and their slight upregulation leads to significantly higher expression of downstream cytokines such as IL-6. The main consequence of increased cytokine production in the bone microenvironment is expansion of the osteoclastic pool because of increased osteoclast formation and their extended lifespan.

Contrary to the evolution of research methods and laboratory tests, in order to identify the associated factors of the chronic periodontitis, the exact prediction of the progression of periodontal disease is still unclear. The evasive nature of the disease is complicated by the fact that different teeth and different sites of the same tooth can present different severity of the disease.

Clinical measurements, such as probing depth, clinical attachment loss or gingival bleeding present limitations in offering a real time evaluation of the disease progression. An ideal instrument for diagnosis would identify not only the presence and severity of the disease, but also could predict the clinical evolution of the inflammation (Kinane et al., 2011).

It is clear that the cytokines work as complex cascade systems and networks in regulating the bone metabolism. Thus, many cytokines can regulate the production of other cytokines and their receptors and, when associated, can exert additive, inhibitory or synergistic effects. Moreover, the complexity is enhanced by soluble and membrane-bound forms of both inhibitory and pro-inflammatory cytokines and their receptors. In another study (Ursarescu et al., 2016), we demonstrated significantly higher values for IL-1 α and IL-1β in osteoporosis patients, when compared to the healthy subjects. There are reasons to

believe that IL-1β can be an important mediator of the gingival and ligament destruction and also of the bone resorption during periodontal disease progression. IL-1β is a strong stimulator of MMP in fibroblasts and ligament cells. IL-1β also stimulates the bone resorption in vivo. The undergoing mechanism involves the RANKL expression in osteoblasts and the indirect stimulation of osteoclast genesis and bone resorption (Lorenzo et al., 2008)

The present study has its limitations regarding the correlations of the cytokine levels with oestrogen levels; supplementary studies are requested to evaluate the effects of local and systemic therapy on a larger scale of cytokines, offering a definite image on the physiopathologic interrelations between chronic periodontitis and osteoporosis.

Conclusions

The periodontal clinical parameters (probing depth, clinical attachment loss and gingival bleeding) and GCF levels of pro-inflammatory cytokines presented higher values for the osteoporosis patients when compared to systemic healthy subjects. The cytokine values were positive correlated to the periodontal clinical parameters. Therefore, these patients are predisposed to an impressive release of such cytokines, with local inflammatory changes which can accelerate the periodontal disease progression. The conventional periodontal therapy generated a significant improvement of the periodontal parameters. The post-therapy serum Il-6 and RANKL values were also low.

Our research demonstrates a strong correlation between the osteoporosis and the periodontal disease; the tight relation between the local factors and the attachment loss is also important. Therefore, the osteoporosis creates favourable systemic conditions for the evolution of the periodontal disease but the former is highly associated with local risk factors.

II.3 Adjunctive therapeutic methods in the complex treatment of periodontal disease

II.3.1 Studies on the chemical control of bacterial plaque

The periodontal disease is considered to be the most common microbial infection in the adult, the studies from the last decades demonstrating the fact that microorganisms are determinant factors in the aetiology of the periodontal disease. From the more than 500 microbial species present in the sub-gingival biofilm, the highest pathogens were identified: *Porphyromonas gingivalis, Treponema denticola* and *Tanerella Forsythia*. They are considered to be the primary etiologic agents which, when interacting to a susceptible host, can initiate inflammatory and destructive processes of the periodontal structures (Umdala et al., 2012).

The main objective of the therapeutic management is to obtain a high standard of oral hygiene and to prevent the loss of periodontal attachment. This objective is possible by an associated action of the patient which has to be motivated to obtain a high standard of oral hygiene and of the medic which presents a high area of mechanical and surgical periodontal procedures. The clinical studies demonstrated that, even when an adequate therapy is conducted, the result can be unfavourable in a certain category of patients. Therefore, it was proved the essential role of the host factors in the onset of the periodontal disease, of the unbalanced local immune response of these patients.

When associated with such systemic conditions, the pathogeny mechanisms of the major periodontal pathogens are the incriminated factors for the existence of aggressive, severe and refractory clinical forms of disease.

In a previous study (Stupu et al., 2014), conducted on a group of 36 patients who underwent subgingival irrigation ultrasonic scaler with a mixture of povidone-iodine and chlorhexidine gluconate, ultrasonic scaling subgingival irrigation with povidone-iodine only

or ultrasonic scaling subgingival irrigation with normal saline solution, we observed that Chlorhexidine proved to be effective in all concentrations (even at 0.05%). Regarding povidone-iodine, active trough inhibited microbial growth was 1%, representing 1/10 dilution compared to the concentration used in general medicine.

This research direction has been materialized by publishing the following papers:
1.Bogdan M, Tica I, Ghorghe DN, Silossi I, Solomon S, Martu I, Surlin P, Chiscop I, Budacu C. Effect of 0.2% Chlorhexidine's use for treatment of localized gingival leions in patients with type 2 diabetes. Rev. Chim (Bucharest) 2016; 67(12): 2651-2653.
http://www.revistadechimie.ro/pdf/BOGDAN%2012%2016.pdf
2.Kappenberg-Nițescu DC, Luchian I, Mârțu I, Solomon SM, Mârțu S, Păsărin L, Mârțu A, Sioustis IA, Goriuc A, Tatarciuc M. Periodontal effects of two innovative oral rinsing substances in oncologic patients. Exp Ther Med. 2021; 21(1): 98.
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II.3.1.1 Effect of 0.2% chlorhexidine's use for treatment of localized gingival lesions in patients with type 2 diabetes

Aim of the study

We conducted a study with the purpose to evaluate the effectiveness of chlorhexidine treatment in localized gingival lesions not related to inflammatory periodontal disease, by assessing the levels of IL1-beta in gingival crevicular fluid (GCF) in healthy patients or with type 2 diabetes.

Materials and method

Eleven subjects were included in the study (Table II.13). The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study. Patients were asked about the presence of diabetes mellitus and smoking.

Table II.13. Demographic data of patients and time from diagnosis (Mean ± SD)

	Healthy		Diabetes	
	Male	Female	Male	Female
Number of patients	3	3	3	2
Age of patients (years)	45.8±3.56		45.8±2.77	
Time from diagnosis (years)	-		3.67±1.21	

Diabetic patients had a good control of their diabetes with blood glucose and HbA1c levels within the normal range. Time from diagnosis was also noted. Patients were conducted ultrasonic scaling with CHX 0.2% as cooling solution and they were recommended rinses of the oral cavity twice a day with mouthwash with CHX 0.2% after dental brushing. Using Periopaper strips, GCF from all patients was collected from the lesion area before treatment and 5 days later. GCF volume was measured with Periotron, diluted in 100 microl PBS and stored at -20^{0}C. IL1beta as inflammatory marker was determined in GCF by ELISA technique using the kit from R&D Systems, USA. Age of patients, time from diagnosis of systemic disease and levels of IL1beta were expressed as mean± SD. Differences between groups were calculated using Mann Whitney U test ($p < 0.05$ being considered statistically significant) and correlations among groups were calculated with Pearson test.

Results

The 11 patients, 6 women and 5 men, had ages between 42-53 years. All patients had

PD<3, without BOP (excepting the affected tooth). According to the presence of the systemic diseases, they were divided in 2 groups: H- 6 patients systemic healthy, D- 5 patients with diabetes (Table C.11). All patients were non-smokers.

Before treatment, in the group D, the levels of IL1beta were higher 1.5 fold than in the group H. The differences were statistically significant ($p<0.05$). After treatment, in the group D, the levels of IL1-beta were higher than in the group H, the differences were statistically significant between D group and H ($p<0.05$). Levels of Il1- beta before and after treatment registered statistically significant differences in all groups (Figure II.17).

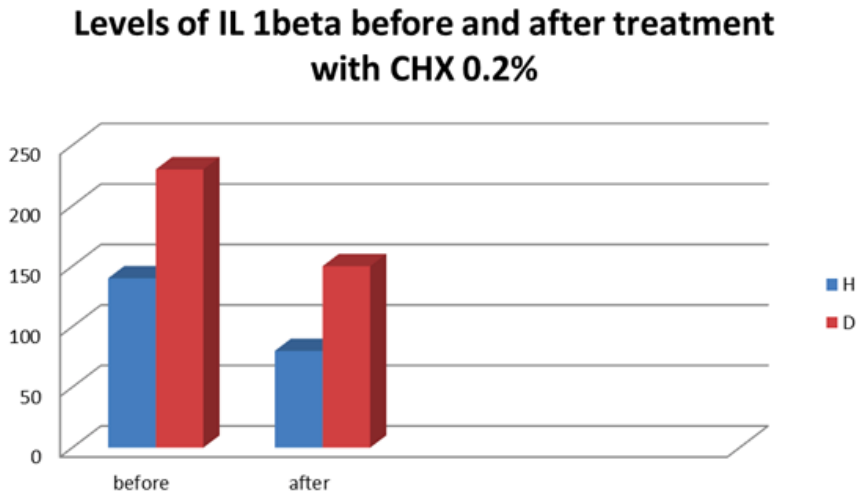

Figure II.17. Levels of IL1beta in all groups before and after treatment (H=group of systemic healthy patients D=group of diabetic patients)

There were found no correlations between the time from diagnosis and the levels of IL1beta.

Discussion

In the review of Van Der Sluijs et al. (2016) for the use of CHX or essential oils, the added effect as coolant in ultrasonics can be considered zero. For povidone-iodine, a very small level of clinical attachment loss (CAL) gain may be expected. One possible explanation could be the fact that the action of the coolant solution is too short to take effect. There are studies showing that the use of CHX chips leads to improved periodontal status and reduced levels of PGE2 in GCF (Ma and Diao, 2020).

Thus, in the present study, it can be considered that the addition of CHX in cooling water of ultrasonics has not influenced the evolution of gum lesion, but patients used CHX mouthwash twice a day and the levels of IL1beta decreased. IL1beta is an inflammatory marker whose levels in GCF were determined both in patients with periodontal disease and in its association with type 2 diabetes (Bulut et al., 2001).

Patients in the present study had no pre-existing periodontal disease but foreign bodies produced lesions cause a strong inflammation with IL1-beta levels higher than those in chronic periodontitis and type 2 diabetes association or chronic periodontitis alone. Before treatment, the levels of IL1-beta were higher in group D, possibly due to the metabolic and inflammatory changes in diabetic condition. These levels remained elevated after achieving treatment with scaling and CHX 0.2% in group D, comparing with other studies for the association of diabetes with periodontitis or periodontal disease alone.

Other studies reported decreases of different biomarkers (e.g. C reactive protein, IL6 etc.) serum levels after first steps of therapy in cases with periodontal disease associated to other systemic diseases like rheumatoid arthritis or atherosclerosis (Boatca et al., 2016;

Solomon et al., 2016).

It could be interesting, in further studies, to assess the levels of different cytokines in serum and GCF of gingival or periodontal lesions associated with other systemic diseases like hepatitis C, while different serum cytokines levels are determined as elevated in patients with hepatitis C-related liver diseases, especially in HCC (hepatocellular carcinoma) patients.

These levels reflect hepatic dysfunction better than liver inflammation parameters, which might explain the higher serum concentrations of cytokines in those patients. It could be interesting, also, to compare the levels of different biomarkers in serum and GCF in periodontal or gingival lesions with those in chronic or aggressive periodontitis alone or associated with systemic diseases.

Conclusions

CHX use in the treatment of gingival lesions produced with foreign bodies leads to IL1beta decreased levels in GCF compared with initial ones and diabetes could negatively influence the evolution of these lesions.

II.3.1.2 **Periodontal effects of two innovative oral rinsing substances in oncologic patients**

Aim of the study

We conducted a research with the aim to evaluate the effects of two antiseptic, antimicrobial and antifungal products on oral cavity and periodontal tissues in oncologic patients during chemotherapy.

Materials and method

The study was conducted on 50 subjects with ages ranging between 48 and 60 years old. The study methodology respected the Helsinki Declaration norms. All patients included in the sample population signed an informed consent prior to being accepted to take part in this study. The total number of subjects included in the present study consisted of 22 females and 28 males, thus 44% were women and 56% were men, the distribution being similar regarding the sex (Table II.14). Most patients were from urban areas (90%) and only a small percentage were from rural areas (10%).

Table II.14. Subject distribution in groups according to the oral antimicrobial, antiseptic and antifungal substance used.

Substance	Absolute frequency	Percentage frequency
Placebo (control)	12	24.0
Oral rinse	22	44.0
Oral coating	16	32.0
Total	50	100.0

Most patients were retired and only 20% were still working or unemployed. The chemotherapy administered to the patients was comprised of cisplatin, oxaliplatin and gemcitabine, the highest frequency of antineoplastic drugs being cisplatin (n=26, 52%), followed by oxaliplatin (n=17, 34%) and gemcitabine (n=7, 14%) (Fig. II.17).

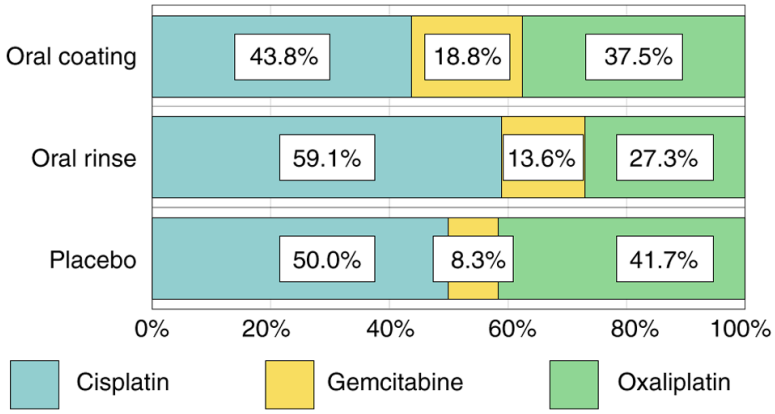

Figure II.17. Distribution of the three groups depending on the chemotherapy agent.

The patients included in the present study suffered from systemic cancer, were undergoing chemotherapy and had a form of periodontal disease. In order to avoid compromising the relevance and validity of the results, the following exclusion criteria were considered: i) tobacco smokers; ii) patients with infectious and/or inflammatory disease that may have affected the periodontal status, with the exception of systemic cancer; iii) patients that had had periodontal treatment in the previous 6 months; iv) patients that had had antibiotherapy or anti-inflammatory treatment in the previous 3 months, with the exception of chemotherapy; vi) patients that used antiseptic oral rinses or medical toothpaste.

All subjects were randomly split into three groups: i) controls, which included chemotherapy patients that did not use any active substance throughout the present study; ii) group A, which included chemotherapy patients that used oral rinses with cetrimide mouthwash three times a day; iii) group B, that included chemotherapy patients that used mouth coating with a pharmacy-made compound two times a day.

The clinical examination considered several elements: probing depth (PD), clinical attachment loss (CAL), dental mobility (M), plaque index and periodontal disease index (PDI) In addition, pathological probing depths higher than 3 mm on teeth with no gingival recessions were considered. The clinical examination took place at two time-points: T0, before beginning to use the active substances and T1, after 14 days of antiseptic, antimicrobial and antifungal substance usage.

The two substances evaluated in the present study were Citrolin oral rinse and an oral coating recipe developed at the pharmaceutical laboratory BabyFarm, Ltd. Citrolin is an oral rinsing solution that contains 25 mg cetrimide, 3 mg lidocaine and excipients per 100 ml of product and is administered in the form of oral rinses 15 ml per rinse, three times a day. The oral coating substance was developed in collaboration with BabyFarm, Ltd. laboratory and its composition contains neomycin, nystatin, metronidazole, sodium bicarbonate, vitamin A, xylene 2% and oleum helianthi.

The three groups were evaluated based on oral hygiene and periodontal status before the commencement of oral rinse use and oral coating and 14 days after use. None of the patients declared any side effects after using the two compounds included in the present study. The statistical analysis of the data included in the present study consisted of descriptive statistics, one Sample t-test and a paired Sample t-test using the SPSS software version 21.0 (IBM Corporation). $P<0.05$ was considered to indicate a statistically significant difference.

Results

The paired Sample t-test revealed a high statistical significance of improvements for group A that used Citrolin oral rinse, the positive modification of all parameters being

statistically significant (P<0.05), with the exception of dental mobility, as revealed in Table II.15.

Table II.15. Statistical significance analysis for group A, between time-point T0 and T1.

Analysed indices	Group A, Citrolin oral rinse T0-T1		
	Mean difference	t	p-value
Silness-Loe Plaque index	0.092	2.358	**0.028**
PDI	0.104	2.097	**0.048**
PBI	0.165	3.578	**0.002**
Mean dental mobility	-0.045	-1.821	0.083
Mean PD	0.145	4.661	**<0.001**
Mean CAL	0.161	3.409	**0.003**
Bold indicates statistical significance. PDI, periodontal disease index; PBI, papillary bleeding index; PD, probing depth; CAL, clinical attachment loss.			

Table II.16. Statistical significance analysis for controls between time-points T0 and T1.

Analysed indices	Placebo group T0-T1		
	Mean difference	t	p-value
Silness-Loe Plaque index	-0.164	-2.680	**0.021**
PDI	-0.472	-4.513	**0.001**
PBI	-0.301	-7.473	**<0.001**
Mean dental mobility	-0.080	-1.948	0.077
Mean PD	-0.479	-4.823	**0.001**
Mean CAL	-1.183	-3.467	**0.005**
Bold indicates statistical significance. PDI, periodontal disease index; PBI, papillary bleeding index; PD, probing depth; CAL, clinical attachment loss.			

The results obtained for the controls revealed an increase in the values of all analyzed indices and periodontal disease progression. The modifications of values for these indices had statistical significance with the exception of the average tooth mobility value that had no statistical significance (P=0.077) (Table II.16). Similarly, group B that used oral coating did not exhibit any improvement of the indices evaluated (data not shown). Moreover, CAL values were different for each of the three chemotherapy agents included in the study between T0 and T1. The average value of CAL was increased in patients treated with oxaliplatin (mean difference=-0.239) and cisplatin (mean difference=-0.19) (data not shown).

Discussion

The efficiency of antineoplastic treatment with platinum-based drugs (cisplatin, oxaliplatin) has been demonstrated multiple times in the past (Zhou et al. 2010; Park et al., 2013), although what does sometimes limit their dosage is their potential side effects. Patients treated with one of these chemotherapy agents may develop up to 40 specific adverse reactions. The most important and frequent effect is nephrotoxicity in the case of cisplatin administration and neurotoxicity in the case of oxaliplatin alongside the well-known myelosuppressive effects (Oun et al., 2018).

Ideally, periodontal disease should be assessed and treated before the beginning of chemotherapy, bearing in mind that a pre-chemotherapy evaluation and maintaining good oral hygiene has been demonstrated to be efficient in preventing oral and systemic complications during anti-neoplasic treatment. Frequent erythematous lesions, ulcerations or candidiasis can occur in the oral cavity during chemotherapy (Napeñas et al., 2007). Moreover, modifications of periodontal parameters can be observed through an increase in the quantity of oral bacterial plaque, an exacerbation of gingival inflammatory signs and even

modifications of the bacterial community composition at oral and periodontal levels (Jensen et al., 2008).

The use of antimicrobial and antiseptic substances is efficient in plaque reduction and improving periodontal parameters. Cetrimide, the active substance in Citrolin, is an antiseptic with multiple quaternary ammonium salts that has a bactericidal effect on a wide spectrum of gram-positive and gram-negative bacteria (Engebretsen et al., 2015). Its action consists of affecting the permeability of the bacterial cellular membrane. It is used in a high number of pharmaceutical compounds with the role to decrease the level of gingival pain and increase oral hygiene (Elzanfaly et al., 2015). It is sometimes used in products that also contain chlorhexidine gluconate (Dostie et al., 2017); however, in the present study, cetrimide was the only active substance in the oral rinse to avoid errors in the results. The use of cetrimide can eliminate bacterial plaque to a great extent, some authors claiming that it has an even higher antimicrobial effect than that of chlorhexidine (Guerreiro-Tanomaru et al., 2014). The effects of cetrimide were also demonstrated to be efficient in preventing carious lesions; a concentration of 0.2% cetrimide used as oral rinse for a minute had the capacity to destroy *Streptococcus mutans* in a proportion of >99% (Ruiz-Linares et al., 2014).

Conversely, presently, there are available substances with topic application that contain either only metronidazole, or neomycin and prednisolone (Moisei et al., 2015). In the present study, we selected to introduce a new compound with topical administration that contained neomycin and metronidazole with the aim of evaluating its periodontal efficiency. This combination of drugs has been used in the past, but in association with general surgery of the digestive tract. The pre-operatory administration was revealed to be an efficient combination of antibiotics that leads to a significant reduction of post-operatory infections (Espin-Basany et al., 2005).

The most important modifications of the Silness-Loe plaque index were observed in the subjects of group A that used oral rinses with cetrimide. The values of the plaque index were decreased after 14 days of using cetrimide and consequently improved the level of oral hygiene. Conversely, higher values in T1 compared to T0 in the control group (2.002 vs. 1.838) were obtained, thus manifesting an increase of 0.164 between the two evaluations. The values of the plaque index in group B were also increased after 14 days (T1=2.055 vs. T0=1.996) (data not shown).

Another fact for consideration is the medullar modifications that occur during chemotherapy that lead to thrombocytopenia, which can be translated into a pronounced tendency to gingival bleeding in the oral cavity, especially in the conditions of a pre-existing periodontal disease (Rapone et al., 2017).

In the study, the PBI exhibited a decrease in value for group A after the 14 days of oral rinsing, signifying an improvement in the periodontal inflammatory status (T1=2.120 vs. T0=2.285; difference =0.165). Then again, controls had higher values of the bleeding index in T1 (T1=2.338 vs. T0=2.037) and group B exhibited similar values at both evaluations (T1=2.206 vs. T0=2.238) (data not shown). These results reflect the modifications obtained for the level of oral hygiene, creating an association between the level of oral hygiene and level of bleeding at the periodontal level.

The average PD obtained in the present study revealed considerable differences between the controls and patients that used antimicrobial/antiseptic/antifungal substances. The highest improvements were observed in group A, which used cetrimide oral rinses. This may be explained by the fact that cetrimide has a higher salivary retention rate than chlorhexidine immediately after the rinse is performed, but decreases more significantly at 4 h than chlorhexidine (Bonesvoll & Gjermo, 1978), which is why the patients were recommended to perform the action of rinsing more often than they would otherwise in order to maintain an optimal concentration in the saliva and at the periodontal level.

Oral mucositis and periodontal disease progression are important modifiers for the level of quality of life of patients undergoing chemotherapy and negatively impact the affective state of patients (Dodd et al., 2001), as we have shown in a previous study (Nitescu et al., 2017).

The present study offers more options regarding the secondary means of oral hygiene that oncology patients can use in order to prevent the progression of periodontal disease and obtain an improved periodontal status during chemotherapy, thus improving their experience during chemotherapy and obtaining an improvement in their level of quality of life (Neagu et al., 2011).

Conclusions

It can thus be concluded that cetrimide oral rinses were demonstrated to be the most efficient secondary means of oral hygiene assessed in the present study. Cetrimide oral rinse decreased the level of bacterial plaque and gingival bleeding and it was efficient in preventing the progression of periodontal disease in patients undergoing chemotherapy.

The present results offer new perspectives regarding a reliable alternative to the contemporary-used secondary means of oral hygiene for oncologic patients undergoing chemotherapy. Thus, the periodontal status of these particular patients can be better controlled and their quality of life can be significantly improved.

II.3.2 The effects of photoactivation therapy on periodontal diseases

State of the art in photodynamic therapy

Photodynamic therapy was discovered accidentally at the beginning of the 20th century and was then applied in the medical field for the light-induced inactivation of cells, microorganisms or molecules (Allison et al., 2006).

The photodynamic therapy involves three major components: the visible light, the oxygen and a nontoxic photosensitizer (a photo-activated substance). The photosensitizer binds to the target cells and is activated by the light source, producing singlet oxygen and other reactive agents, highly toxic to bacteria (Takasaki et al., 2009). The excited singlet oxygen can oxidize many biological molecules (proteins, nucleic acids and lipids), leading to its cytotoxicity. Singlet oxygen has a diffusion distance of approximately 100nm and a half-life of <0.04 ls (Soukos & Goodson, 2011).

The photodynamic activity is influenced by the type, the dose, the incubation time and the localization of the photosensitizer, the wavelength of the light source (nm), the light power density (mW/cm2) and the light energy flow (J/cm2). In this type of therapy, the toxic effect is mainly due to the damage of the cytoplasmic membrane and of the DNA (Kikuchi et al., 2015).

The ideal photosensitizer should present the following properties: a high quantum yield of triplet state to obtain large concentrations of the activated drug; a high singlet oxygen quantum yield; high binding affinity for microorganisms; a broad spectrum of action; low binding affinity for mammalian cells to avoid the risk of photo-destruction of host tissues; a low propensity for selecting resistant bacterial strains; a minimal risk of promoting mutagenic processes; and low chemical toxicity (Kikuchi et al., 2015).

In antimicrobial photodynamic therapy, the frequently used photosensitizers are toluidine blue O, methylene blue, erythrosine, chlorine e6 and hematoporphyrin, which have been shown to be safe when employed in the medical field.

This research direction has been materialized by publishing the following papers:

1.Solomon S, Ursarescu I, Martu A, Luchian I, Agop-Forna D, Martu S, Forna NC. Photo-activated toluidine Blue O as adjunctive periodontal treatment. REV. CHIM (Bucharest) 2015; 66(8):1166-1168.
http://www.revistadechimie.ro/pdf/SOLOMON%20S.pdf%208%2015.pdf
2.Nicolae V, Matei MN, Ibric Cioranu VS, Martu MA, Luchian AI, Martu S, Solomon SM. The use of photoactivated Blue-O toluidine for periimplantitis treatment in patients with periodontal disease. Rev. Chim (Bucharest) 2015; 66(12): 2121-2123
http://www.revistadechimie.ro/pdf/NICOLAE%20VASILE%2012%2015.pdf

II.3.2.1 **Photo-activated toluidine blue O as adjunctive periodontal treatment**

Aim of the study

The purpose of our study was to assess the clinical efficiency of the adjunctive photodynamic therapy in the etiologic periodontal treatment, associated to classic mechanical bacterial plaque removal.

Materials and method

We recruited a number of 72 patients with chronic periodontitis, divided in two groups: the study group and the control group. The patients with periodontal therapy in the last 12 months or with antibiotic therapy in the last 6 months, patients with inflammatory or infectious systemic diseases, patients taking various types of drugs which can affect the periodontal status and smokers were excluded from the study. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

Each subject received a rigorous clinical examination; the periodontal clinical indexes (bleeding on probing, periodontal pocket depth, clinical attachment loss) were recorded; all the measurements were conducted with the aid of periodontal probes (Williams). The bleeding on probing was quantified as follows: 0 (no gingival bleeding), 1 (point of bleeding), 2 (linear bleeding), 3 (triangular bleeding) and 4 (drop of blood).

The probing depth was registered for all the teeth present on the dental arch, measured in six points per tooth (mesial-facial, middle-facial, distal-facial, mesial-oral, middle-oral, distal-oral), from the free gingival margin to the base of the pocket; the measurements higher than 3mm per site were considered as pathological.

The clinical attachment loss was measured from the cementum-enamel junction to the base of the periodontal pocket. The periodontal diagnosis was set after the completion of the clinical examination.

The method for dividing the groups was randomized. The study group received etiologic therapy (supra- and subgingival scaling, root planning, professional brushing), followed by photo-activated disinfection of the periodontal pockets (LED PAD therapy). The control group received only etiological standard therapy (supra- and sub-gingival scaling, root planning, professional brushing), without photo-activated disinfection.

The LED source used in this study was in the red spectrum (wavelength of 635nm, Denfotex UK) and a viscous solution of tolonium chloride 0.01mg/mL provided by the manufacturer served as a photo-sensitizer (Denfotex UK). We followed all the steps from the operatory protocol, according to the manufacturer's recommendations. After the tooth isolation, the photo-sensitizer was meticulously placed in the periodontal pockets, followed by a LED irradiation for 60 s. For the periodontal pockets with a depth higher than 5 mm we used a special Perio-tip for the light source. During the photo-activation we used protection goggles for the protection of the patient and of the medic. The PAD therapy was repeated at

7, 14 and 21 days from the first session. The patients were recalled after 2 months for re-assessment. The periodontal clinical parameters were also re-assessed.

The baseline and after two months data were registered and statistically analysed; for the statistical analysis we used the Microsoft Excel 2010 and PASW 18 Statistics soft-wares.

Results

The 72 subjects were divided in two groups: the study group (n = 35) and the control group (n = 37). The subjects included 48 males and 24 females.

The age of the subjects in the study group ranged between 31 and 75 years old (with a mean value of 47.3 ± 3.9 years old) and in the control group, between 36 and 68 years old (with a mean value of 49.6 ± 2.8 years old). The demographic data are summarized in Table II.16.

Table II.16. Demographic data of the study and the control group

Parameter		Study Group	Control Group
Age (years)	Interval	31-75	36-68
	Mean	47.3±3.9	49.6±2.8
Gender	Male	22	26
	Female	14	10
Provenience environment	Urban	26	23
	Rural	9	14
Number of sites with classic therapy		521	552
Number of sites with adjunctive therapy		521	0

Table II.17. Changes of clinical parameters of the study and the control group

Parameter	Study Group	Control Group	p-value
Bleeding on probing	68%	53%	<0.03
Pocket depth (mm)	1.24	0.57	<0.05
Gain of clinical attachment (mm)	0.37	0.14	<0.04

After the 2 months examinations we observed a significant improvement of the periodontal parameters. Decreased values for the probing depth and for the BOP were noticed for both groups, with higher differences for the study group than the control group. We also remarked a gain of periodontal clinical attachment, more significant for the PAD group. The clinical statistic results are presented in Table II.17.

Discussion

The results of our study support a series of published data reporting positive results of the photo-activation therapy. Improved results were observed in a study using PAD with methylene blue on chronic periodontitis patients (Segarra-Vidal et al., 2017). The same favourable results were obtained in another study with LED PAD and phenothiazine chloride (Alvarenga et al., 2019).

Our re-evaluation results contradict other studies which did not observe a clear advantage of laser PAD in chronic periodontitis patients. A study conducted on patients with chronic periodontitis who received only one session of LED PAD and toluidine blue therapy did not reveal significant changes of the clinical parameters (Theodoro et al., 2011). Another study demonstrated that PAD therapy reduces a series of clinical parameters but not the bleeding on probing, demonstrating a non-significant diminishing of the inflammation degree (Bassir et al., 2013).

The optimal parameters required for effective antimicrobial photodynamic therapy-induced killing of supra gingival periodontal pathogens using the combination of different

toluidine blue O concentrations and laser-irradiation energies were investigated and reported that diode laser irradiation at 12 JD cm2 with 1 mgD mL of toluidine blue O was the most effective option (Qin et al., 2008).

The differences between the various studies results can be explained by the different study designs, by the different types of activation sources and by the high variety of photosensitizers.

Conclusions

The photo-activated disinfection therapy of the periodontal pockets proves itself as a viable adjunctive method to the classical mechanical plaque removal in the chronic periodontitis patients; the LED source is also less aggressive than the usual laser ones, providing a safer and more accessible method and the association of the toluidine blue O determines significant improvement of the periodontal clinical parameters (bleeding on probing, clinical attachment loss and pocket depths).

II.3.2.2 **The use of photoactivated toluidine blue O for periimplantitis treatment**

Aim of the study

The aim of the study was to assess the effects of the photodynamic therapy on the periodontal clinical parameters in patients with peri-implantitis, as an adjunctive method to the classical mechanical treatment.

Materials and method

We recruited a number of 44 patients, with dental implants and peri-implantitis, randomly assigned to two groups: the study group and the control group. The inclusion criteria were represented by the presence of probing pocket depths (PPD) between 4 and 6mm. the exclusion criteria comprised any systemic disease which could influence the peri-implant tissues (inflammatory and infectious diseases), smoking, use of antibiotics/anti-inflammatory drug in the past 6 months, periodontal treatment in the last 6 months, pregnancy/lactation, allergy to toluidine blue. The study methodology respected the Helsinki Declaration norms and the signed informed consent was obtained from each subject included in the study.

Each patient was clinically examined and the following parameters were registered at baseline and at 3 months after the treatment protocol:

- probing pocket depth (PPD) was assessed as the primary outcome following intervention using a William's graduated periodontal probe at six inter-dental sites (mesiobuccal, buccal, disto-buccal, mesio-oral, oral and disto-oral);
- clinical attachment levels (CAL), gingival index (GI), gingival bleeding index (GBI) and plaque index (PI) were assessed before and after treatment as secondary outcomes.

All the 44 subjects were treated by an experienced specialist and clinical outcomes were measured by another specialist who was blinded to patient selection. The control group was administered scaling and root planing (SRP) by carbon fiber hand scalers and Gracey curettes (Hu-Friedy).

No other treatment was given to this group. Full-mouth supragingival and subgingival scaling was performed at all sites within 24 h. This group included 22 subjects (19 women and 5 men; mean age: 38.4 ± 9.6 years).

The test group included 22 subjects (11 women and 11 men; mean age: 40.8 ± 8.3 years) and was managed by a photo-activated disinfection treatment (PDT) in addition to SRP. The LED source used in this study was in the red spectrum (wavelenght of 635nm, Denfotex UK) and a viscous solution of tolonium chloride 0.01mg/mL provided by the

manufacturer served as a photo-sensitizer (Denfotex UK). We followed all the steps from the operatory protocol, according to the manufacturer's recommendations. After the isolation of the site, the photo-sensitizer was meticulously placed in the peri-implantary pockets, followed by a LED irradiation for 60 s. For the pockets with a depth higher than 5 mm we used a special Perio-tip for the light source.

A subject-level analysis was performed statistically for each of the parameters using SPSS software for Windows, Version 16.0. Median (minimum to maximum; interquartile range) for the clinical variables were calculated for each treatment. Significant difference between the test and control groups with respect to categorical data was assessed using Chi-square test, whereas Mann-Whitney U-test was used for continuous variable. Likewise, Wilcoxon's Signed Rank Test was used for finding significant changes from baseline to various intervals within the test and control groups.

Results

We examined and treated a number of 44 subjects, divided in two groups: the study group (n=22, 19 women and 5 men; mean age: 38.4 ± 9.6 years) and the control group (n=22, 11 women and 11 men; mean age: 40.8 ± 8.3 years).

No significant differences were found between the test and control group of patients with regard to the baseline values of clinical parameters ($p > 0.05$), except for the plaque index ($p < 0.01$) (Table II.18).

As compared to control group, PPD and CAL showed statistically significant reduction in the test group at 3 months ($p < 0.05$).

A statistically significant improvement in gingival index and gingival bleeding index was seen for the study group ($p < 0.01$) after 3 months of PDT, whereas the difference in plaque index was above the significance level ($p > 0.05$).

In our study, SRP was given to both the two groups as it would be unethical to deny the conventional treatment to anyone. When compared with baseline data, PPD showed higher improvement in the test group than control group at recall visits of 3 months. Statistically significant reduction in GI was observed in the test group as compared to the control group after 3 months of PDT ($p < 0.01$). PI also showed significant reduction for the study group ($p < 0.05$).

Table II.18. Differences in clinical parameters between group and within the groups, when compared at baseline, after 3 months

Parameter	Baseline			At 3 months			p-value (baseline/ 3 months)	
	Study group	Control group	p-value	Study group	Control group	p-value	Study group	Control group
PPD (mm)	5.8 (5.0-6.0, 1.0)	5.6 (4.4-6.0, 1.0)	0.363	3.0 (2.0-6.0, 1.0)	4.0 (2.0-6.0, 1.0)	0.016	<0.01	<0.01
CAL (mm)	6.7 (5.2-8.0, 1.4)	6.2 (4.4-8.0, 1.7)	0.455	4.0 (2.6-7.0, 2.0)	4.5 (2.0-7.0, 2.0)	0.021	<0.01	<0.01
GI	2.2 (1.7-3.2, 0.8)	1.4 (0.7-3.2, 1.0)	0.904	0.5 (0..0-2.5, 1.0)	0.5 (0.0-1.5, 0.7)	0.426	<0.01	<0.01
PI	2.2 (0.7-3.2, 0.8)	1.4 (0.7-3.2, 1.0)	0.001	0.5 (0.0-2.5, 1.0)	0.5 (0.0-1.5, 0.7)	0.426	<0.01	<0.01
GBI	100 (50.0-100.0, 25.0)	75 (50.0-100.0, 25.0)	0.232	25 (0.0-100.0, 50.0)	50.0 (0.0-100.0, 75.0)	<0.01	<0.01	<0.01
Values are expressed as Median (Min-Max, inter-quartile range); PPD: periodontal probing depth; CAL: clinical attachment level; GI: Gingival Index; PI: Plaque Index; GBI: Gingival Bleeding Index								

Another notable effect was the statistically significant reduction in GBI in the test group after 3 months ($p < 0.01$).

Discussion

The results of this study support the fact that PDT has a positive effect on patient care, mainly due to the considerably fast resolution of overt inflammation in the gingival tissues, which is supported by the significant reduction in GI, PI and GBI. A plausible explanation for improvement in GI and GBI in test group patients could be due to bacterial load reduction and inactivation of bacterial virulence factors and cytokines when the toluidine blue is irradiated.

In a clinical case-series study, Haas et al. (2000) investigated the clinical effects of treatment of antimicrobial photodynamic therapy (toludine blue O + diode laser) in combination with guided bone regeneration using autogenous bone grafts on 24 implants diagnosed with peri-implantitis in 17 patients. They reported that 21 implants out of 24 showed improvements in the bone defect after a mean observation period of 9.5 months. In a case report Schuckert et al. (2006) demonstrated effective bone regeneration within bone defects around implants affected by peri-implantitis following surgical therapy using photodynamic therapy (tolonium chlorine + 100 mW diode laser) to decontaminate the implant surface and the application of recombinant human bone morphogenetic protein-2.

Conclusions

The LED photo-activated adjunctive therapy with toluidine blue O as a photo-sensitizer exerted an important role in the improvement of the clinical parameters in patients with peri-implantitis, when compared to the simple usage of conventional SRP therapy, with significant reduction of the local inflammation and of the pocket depths.

Moreover, LED therapy is less expensive than laser as a light source, making this method a more accessible one to the large usage. Our results support the clinical usage of LED photo-activated therapy as an effective option in the etiological treatment of compromised implants.

II.3.3 Antibiotics in the adjunctive treatment of periodontal disease

Antibiotics represent an important adjunctive periodontal therapy. This research direction has been materialized by publishing the following papers:

1.Moisei M, Pasarin L, Solomon S, Oanta C, Tatarciuc D, Ursarescu I, Martu S. The role of antibiotherapy in the oral rehabilitation of the periodontal affected patient. Rom J Oral Rehab 2015; 7(1): 107-112.
http://www.rjor.ro/the-role-of-antibiotherapy-in-the-oral-rehabilitation-of-the-periodontal-affected-patient/?lang=ro

2.Ursărescu IG, Păsărin L, Solomon SM, Cioloca D, Nițescu DC, Boatcă M, Mârțu S. The effects of the adjunctive therapy with subantimicrobial doses of doxycycline in patients with osteoporosis and chronic periodontitis. Rom J Oral Rehab 2015; 7(2): 56-61.
http://www.rjor.ro/the-effects-of-the-adjunctive-therapy-with-subantimicrobial-doses-of-doxycycline-in-patients-with-osteoporosis-and-chronic-periodontitis/?lang=ro

3.Zaharescu A, Martu I, Luchian AI, Martu MA, Sufaru IG, Martu C, Solomon SM. Role of adjunctive therapy with subantimicrobial doses of doxycycline in glycemic control (HbA1c) in patients with diabetes and endo-periodontal lesions to prevent sinus complications. Exp Ther Med. 2021; 21: 277.
https://www.spandidos-publications.com/10.3892/etm.2021.9708

II.3.3.1 Antibacterial dosage of antibiotic

The drug adjunctive therapy imposed itself as an absolute necessity. The antibiotics played a major role in this therapy, justified more by the existence of a remaining flora in the periodontal structures and the presence of co-morbidities in the systemic status of the patient.

The studies demonstrated the efficiency of the antibiotics in the treatment of the periodontal disease by improved clinical and biologic parameters. Reduced inflammation, reduced probing depths (0,2-0,3mm), improved clinical attachment level and low red complex bacteria were demonstrated (Kapoor et al., 20012). Table II.19 systematize the indications of the antibiotic treatment.

Table II.19. Systemic administration of antibiotics: selection criteria and indications

Selection criteria	Indications
According to the clinical form of periodontal disease	1.ANUP and ANUG with local, regional and systemic complications 2.Aggressive periodontitis 3.Severe chronic periodontitis 4.Refractory periodontitis
According to the systemic status of the patient	Prophylaxis in patients with systemic impairment (in order to prevent bacteraemia)
According to the therapy management	Surgical treatment Non-surgical treatment Membrane use

In order to obtain a favourable therapy response, the selection of the type of the antibiotic is conducted considering the following aspects:

1. Factors regarding the antibiotic: action spectrum, type of activity (bactericide or bacteriostatic), pharmacokinetic profile, adverse effects, administration method, proofs of clinical efficiency.
2. Patient related factors:
   A. History of antibiotherapy for the periodontal disease: self-medication, incorrect therapy (period, association with other drugs), antibiotherapy for other diseases, hypersensitivity phenomena.
   B. The age of the patient: some antibiotics need to be avoided in the period of tissue development (tetracycline accumulates in the bone and tooth tissues in this period).
   C. High risk co-morbidities, like hepatic and renal maladies; in these cases the lowest toxic level antibiotics are selected, with dose adjustments.
   D. The presence of certain physiologic conditions – pregnancy imposes to avoid teratogenous risk drugs; the antibiotherapy is conducted only if the situation strictly requires it and it is based also on an inter-disciplinary consult.
   E. Drug therapies with considerable interactions with antibiotics (Table II.20).

From the large variety of antibiotics, the antibiotics with the maximum efficiency on periodontal tissues were identified by research studies. The main criteria used in the assessment of the antibiotic efficiency involved their antimicrobial activity, defined as a rapport between the maximum concentration in the gingival crevicular fluid (GCFC) and the minimal inhibitory concentration (MIC90), result which is expressed in percentage for each antibiotic and each microorganism.

GCFC offers information regarding the maximum levels reached by the antibiotic in the primary ecological site on systemic intake. MIC90 represents an in vitro determination of the concentration which generates an inhibition of 90% of the bacterial development in a single species.

Based on this determination, it was demonstrated that the most efficient antibiotic for the treatment of a certain periodontal pathogen is the one where the rapport is ≥100% (Krayer et al., 2010).

Table II.20. Interactions between antibiotics with other drug classes

ANTIBIOTIC	OTHER DRUGS	EFFECT
AMOXYCILLIN	PROBENECIDE	It rises the amoxycillin
METRONIDAZOLE	BARBITURICS; HYDANTOIN	It lowers the effect
	ORAL ANTICOAGULANTS	It rises the anticoagulant effect
	ETHANOL	Anti-abuse effect
TETRACYCLINE	Al, Bi, Fe, Mg ANTIACIDS	Lowers the absorbtion level
	BARBITURICS; HYDANTOIN	Lowers the serum concentration
	CARBAMAZEPINE	Lowers the serum concentration
	DIGOXIN	Rises the digoxin level in serum
ERYTHROMICIN AZYTHROMICIN CLARITHROMICINE	CARBAMAZEPINE	It rises the level of carbamazepine in serum with nistagmus, nausea, vomit, ataxia
CPROFLOXACIN	ORAL ANTICOAGULANTS	It rises the anticoagulant effect
	NONSTEROIDAL ANTIINFLAMATORY DRUGS	Rises the risk of CNS obnubilation
	CAFFEINE	Rises the caffeine concentration

Table II.21 summarizes the antibiotics and their activity mechanism (Oettinger-Barak et al., 2013).

Frequently, in the periodontal disease the systemic antibiotherapy uses associations. The advantages of the associations consist in the extension of the microbial range, the prevention/exclusion of bacterial resistance and a decrease of the individual dosage by exploiting the synergic effect. The most frequent association are with metronidazole, except doxycycline (Feres & Socransky, 2012).

Table II.21. The administration of antibiotics and their activity mechanism

ANTIBIOTIC	CLINICAL FORM	DOSE	TREATMENT	ACTIVITY MECHANISM
AMOXYCILLIN (bactericide)	Severe chronic periodontitis	500 mg x3/day	8 days	Inhibits the synthesis of cell wall
	Periodontal abscess with local-regional complications		3 days	
	ANUG		7 days	
DOXYCYCLINE	Localized aggressive periodontitis	Ist Day: 100mg x2/day From IInd Day: 100 mg/day	7-14 days	Inhibits the protein synthesis
CLINDAMYCINE (bactericide)	Periodontal abscess with local-regional complications	600 mg x 3 /day	5 days	Inhibits the protein synthesis
	Localized aggressive periodontitis	300 mg x 3 /day or 600 mg x 2 /day	8 days	
CIPROFLOXACIN (bactericide)	Severe chronic periodontitis	500 mg x 3 /day	8 days, 8-14 days in smokers	Inhibits the nucleic acids synthesis
ZINNAT (bactericide)	Severe chronic periodontitis, ANUG, ANUP Periodontal abscess	250 mg x 2/day	7 days	Inhibits the synthesis of cell wall

	with local-regional complications			
RODOGYL (bactericide)	ANUP	4-6 tb /day	5 days	Inhibits the protein synthesis
AUGMENTIN (bactericide)	Severe chronic periodontitis	1g x2/day	8 days	Inhibits the synthesis of cell wall
	ANUP		7-10 days	
METRONIDAZOLE (bactericide)	Severe chronic periodontitis	500mg x 3/day	8 days	Inhibits the nucleic acids synthesis
	Periodontal abscess with local-regional complications		3 days	
	ANUG		3-7 days	
	ANUP		7-10 days	
AZYTHROMYCINE (bactericide)	Localized aggressive periodontitis	250 mg x2-1st day, then 250mg/day	5 days	Interacts with phagocytosis
	Periodontal abscess with local-regional complications	500 mg x 2- Ist day, then 500 mg x 3 /day	4 days	
ROVAMICINE (bactericide)	ANUG	1.500.000 x 3 /zi 3.000.000 x 2 /zi	7 days	Inhibits the protein synthesis
	ANUP	1.500.000 x 3 /zi 3.000.000 x 2 /zi	7-10 days	

The prophylaxis with antibiotics is recommended in patients with an affected immune response due to other systemic diseases (cardiovascular, diabetes mellitus, immunosupression) (Table II.22).

The systemic therapy with antibiotics presents the following advantages: it is not limited to just one site, exerts an action to all the biologic sites (tonsils, dorsal surface of the tongue, gingival cap of the wisdom molar), the intake is easy (when oral), the extensive antimicrobial action reduces the risk of re-infection, prevents the relapse and it selectively focuses on periodontal tissues (tetracycline is the most potent) (Matarazzo et al., 2008).

In a previous study we demonstrated that patients receiving antibiotics as an initial therapy demonstrated significant improvements, comparatively with those who were given such treatments after the etiological one. Both treatments optimized the average PPD and LCAL values, their positive modification continuing, so that the maximum level was attained 8 months after the treatment.

Table II.22. Prophylaxis with antibiotics in systemic diseases

ANTIBIOTIC	DOSAGE FOR ADULT	DOSAGE FOR CHILDREN
AMOXYCILLIN (oral) AMPICILLIN (parenteral)	2g before the procedure and 1,5g after 6hrs 2g im/iv 30 minutes before the procedure	50mg/kg 1h before 50mg/kg im/iv 30 before
Allergic to penicillins CLINDAMYCINE or AZYTHROMYCINE CLARITROMYCINE (oral) CLINDAMYCINE (parenteral) VANCOMYCINE (parenteral)	600mg 1h before 500mg 1h before 600mg iv 30minutes before 1g 1h before	20mg/kg 1h before 15mg/kg 1h before 20mg/kg iv 30min before 20mg/kg iv 1h before
Immediate hypersensibility to penicillins CEPHASOLYN	1g iv 30min before	25mg/kg 30min before

The disadvantages of the systemic therapy with antibiotics consist in secondary effects which can be difficult to deal with (candidiasis, gastro-intestinal troubles, allergies, risk of resistant species), high hepatic or renal toxicity for some of the antibiotics, drug interactions and different concentrations for different drugs. The local antibiotherapy is indicated when there are maximum 3 sites with periodontal pockets deeper than 5-6mm. It presents the advantage that the slow liberation on longer periods does not generate systemic secondary effects but it has the disadvantage that it does not reach all the biologic sites. Table II.23 summarizes the local antibiotic systems.

Table II.23. Local antibiotic systems

Presentation form	Drug
Cream	DONTISOLON -neomycin, prednisolon DENTOMICIN - microcyclin 2%
Gel	ATRIGEL - tetracyclin SURGIGEL- tetracyclin ELYZOL- metronidazole 25%
Semi-solid systems	SISTEM EVA- fibers of tetracycline of 0,25 mg/mm, non-degradable ACTISITE- tetracyclin 20% copolimer nonresorbable
Biodegradable systems	PERIOCHIPS-soluble membrane with chlorhexidine, active for 7 days

Table II.24 presents the differences between the systemic and local delivery systems of antibiotics.

Table II.24. Systemic antibiotherapy versus local antibiotherapy

Results	Systemic delivery	Local delivery
Distribution	Large distribution	Reduced efficiency range
Concentration	Different levels in different organs and systems	High dosage in situ, low level in the rest of the sites
Therapeutic potential	Can reach a larger variety of distribution for microorganisms	Can exert a better local action
Issues	Systemic adverse effects	Re-infection by untreated sites
Clinical limits	Requires a good patient compliance	Limited infection in the treated site
Diagnosis issues	Identification of pathogens, drug selection	Model of lesion and pathogen distribution, identification of the sites which need to be treated

II.3.3.2 The host-modulation therapy with sub-antimicrobial doses of doxycycline

State of the art in the SDD modulation therapy

The periodontal treatment was mainly focused along the time on reducing the bacterial loading and disorganizing the biofilm by mechanical methods. Still, recent research led to a shift in the concept of the evolution of periodontal disease. Therefore, today it is well known that the lesions of superficial and profound periodontal tissues are a result of the immune and inflammatory defence mechanisms of the host. It is clear that the proinflammatory mediators and the cytokines which are produced by the host cells, along with the proteolythical enzymes (like matriceal metalloproteinases - MMPs), have a significant role in the onset and evolution of the periodontal disease. These effects, especially those exerted on bone tissue, a result of the activation of the RANK/RANKL axis, are even more profound in cases of systemic impairment (like osteoporosis).

The importance of the inflammatory response of the host in the periodontal disease allows the opportunity to explore new therapeutic strategies by means of host response

modulation. The modulation therapy can be associated to classical therapy with the main purpose of reducing the bacterial and inflammatory loading.

Up until now, the only approved systemic therapy of host response modulation in the periodontal disease is the therapy with sub-antimicrobial doses of doxycycline (Periostat®) (SDD), which inhibit the MMP activity.

Great benefit from this type of therapy was observed in a study conducted in 2015 by Ursarescu et al., on 26 patients with osteoporosis. The patients were randomly divided in two groups: study group (n=13), with classical debridement therapy (scaling and root planing) plus sub-antimicrobial doses of doxycycline (20mg twice a day), for 3 months and the control group (n=17), on which only classical debridement therapy was performed.

There was not a significant difference between groups at baseline concerning the probing depth.

After the SDD therapy the moderate and profound sites demonstrated significant attachment improvements at 3 and 6 months, when compared to baseline ($p<0.025$). The superficial sites did not present significant changes throughout the study period ($p>0.05$).

The superficial sites in the control group presented a slight attachment loss (-0.04mm at 3 months, -0.03mm at 6 months). On the other hand, the superficial sites in the study group manifested a slight attachment gain, but without reaching a significant level between groups. Even though the attachment gain in moderate and profound sites was higher for the study group (1.12mm) when compared to the control group (0.78mm), there was not a significant difference ($p>0.05$).

Doxycycline contributes to lowering the connective destruction by inhibiting the proinflammatory mediators (including IL-1 and TNFα), and by an uprising collagen production, of the osteoblast formation and bone formation; the latter aspect is of major importance particularly for the osteoporosis patient, on whom the bone mass is affected. In our study the majority of patients were of female gender; this fact supports the literature data, which present a higher frequency of osteoporosis in female patients than in male patients.

II.3.3.2.1 Role of adjunctive therapy with subantimicrobial doses of doxycycline in glycemic control (HbA1c) in patients with diabetes and endo-periodontal lesions to prevent sinus complications

Aim of the study

The purpose of the study was to analyze local and regional changes (in terms of odontogenic sinusitis) in subjects with endo-periodontal lesions and diabetes mellitus and to investigate the effect on the level of glycemic control (glycated hemoglobin) that could be generated by adjunctive therapy with subantimicrobial doses of doxycycline.

Materials and method

Patients. This study was performed on 51 subjects with diabetes mellitus type 2, divided into two therapeutic groups: 31 patients with diabetes (the SDD group) who underwent conventional endo-periodontal therapy and subantimicrobial doses of doxycycline and 20 patients with diabetes who followed only conventional endo-periodontal therapy (the control group). All of these patients had endo-periodontal lesions.

We excluded from the study patients with systemic diseases that are not a complication of diabetes mellitus, patients suffering from cancer, pregnant, breastfeeding or menopausal women, smokers, patients receiving dental treatment in the last 12 months or standard antibiotic regime in the last 2 months and those who had less than 20 remaining teeth.

In conducting the research, the ethical norms set out in the Declaration of Helsinki were respected. The present study was approved by the Ethics Committee of "Grigore T.

Popa" University of Medicine and Pharmacy (Iasi, Romania). Patients were informed about the aim of the study and each signed the informed consent required for study enrollment.

Clinical examination. Patients underwent a complex endodontic and periodontal clinical examination, which comprised vitality tests and the determination of the following periodontal parameters: probing depth (PD), clinical periodontal attachment loss (CAL) and bleeding on probing (BOP).

The periodontal probing was performed with both the manual periodontal probe (CP-12, Hu-Friedy Mfg. Co., LLC) and an electronic one (Pa-On, Orange Dental GmbH & Co., Germany). The probing depth, together with the loss of periodontal attachment, were measured in six points per tooth: mesial-vestibular, central-vestibular, distal-vestibular, mesial-oral, central-oral, distal-oral. The BOP index was assessed by examination after 30 sec of each probed site. Clinical examinations were conducted at baseline (T0), and at 3, 6 and 12 months from baseline (T1, T2 and T3, respectively). All the data collected from the periodontal measurements are included in the patient's individual periogram.

Clinical examinations were supplemented with serial retro-dental-alveolar radiographs and CBCT examinations for the areas indicating radiological signs of odontogenic sinusitis.

Therapeutic procedure. All patients underwent non-surgical periodontal therapy, consisting of manual and ultrasonic scaling and root planning, with the help of curettes (Hu-Friedy Mfg. Co., LLC). For the mechanical instrumentation of root canals, the access cavity was made, the canals were permeabilized with Kerr needles (Kerr Corp., USA) no. 10 or 15. The instrumentation was performed with the manual ProTaper system (Dentsply Sirona, USA), using the crown-down technique. Each patient was trained on the appropriate oral hygiene means.

These therapeutic procedures were performed for all subjects included in the study. In addition, patients in the SDD group underwent adjunct therapy to modulate the host's inflammatory response to bacterial aggression with subantimicrobial doses of doxycycline, 20 mg twice daily, for 3 months. Adverse events were monitored and recorded throughout the study.

Each subject performed, at home, oral rinses with 0.10% chlorhexidine solution and 0.50% chlorobutanol (Eludril®), twice/daily after dental brushing, for 2 weeks, starting on the first day of endo-periodontal treatment.

For patients with poor glycemic control, infection prophylaxis was also performed, with oral amoxicillin 2 g, taken as a single dose, 1 h before each treatment session. The patients that required this type of prophylaxis treatment were excluded from the study.

Analysis of glycated hemoglobin. For each patient, glycated hemoglobin A1c (HbA1c) was determined. The method of determining HbA1c was immunoturbidimetric. This test does not interfere with other forms of pathological hemoglobin, such as carbamylated hemoglobin in uremic patients or acetylated hemoglobin caused by aspirin treatment; this is due to the high specificity of the anti-HbA1c antibodies for a 4 amino acid sequence at the N-terminus of the β chain in the glycated state. Therefore, this test determines "real" HbA1c, as defined by the International Federation of Clinical Chemistry (IFCC) (Groche et al., 2003).

The quantification of glycated hemoglobin in total hemolyzed blood was based on a turbidimetric inhibition reaction. In a first step, glycated hemoglobin from the collected sample reacts with anti-HbA1c antibodies, with the formation of soluble antigen-antibody complexes. In the second step, polyhaptenes are added, which react with excess anti-HbA1c, by forming antibody-polyhaptene complexes, which are determined by immunoturbidimetry. The total hemoglobin concentration is determined in a separate channel. In the hemolyzed blood sample, the released haemoglobin is converted into a derivative, with a characteristic absorption spectrum; it is measured in two colors.

The percentage calculation of glycated haemoglobin is performed according to the Diabetes Controls and Complications Trial/National Glycohemoglobin Standardization Program (DCCT/NGSP) protocol **(**Little et al., 2001**),** to which a correction formula is applied: % HbA1c = (HbA1c/Hb) x 91.5 + 2.15. This evaluation was performed at the beginning of the study and 3, 6 and 12 months after baseline.

Statistical analysis. The data obtained during the course and at the end of the 12 months of the study were analyzed and statistically processed. The average values for the bleeding index, the probing depth and the level of clinical attachment loss per patient and at group and subgroup level were calculated. The Mann-Whitney test was used in order to detect significant differences between groups at different time points. The Wilcoxon test was used to evaluate changes over time. Values of $P<0.025$ were considered statistically significant. The Mann-Whitney test with a significance level $P<0.05$ was used to determine the significant differences between groups.

Results

The mean age of the 51 subjects was 52.97±10.21 years. The group consisted of 30 female subjects (58.82%) and 21 male subjects (41.18%). Regarding the environment of origin, 33 subjects came from urban areas (64.71%) and 18 from rural areas (35.29%). Demographic data by study group are presented in Table II.25.

Table II.25. Demographic data of the study groups.

Parameters	SDD group (n=31)	Control group (n=20)	Total (n=51)
Age (years) (mean ± SD)	52.17±9.72	53.23±8.38	52.97±10.21
Sex, n (%)			
Male	13 (41.94%)	8 (40.00%)	21 (41.18%)
Female	18 (58.06%)	12 (60.00%)	30 (58.82%)
Provenance, n (%)			
Urban	21 (67.74%)	12 (60.00%)	33 (64.71%)
Rural	10 (32.26%)	8 (40.00%)	18 (35.29%)
Odontogenic sinusitis prevalence, n (%)	18 (58.06%)	11 (5.00%)	29 (56.86%)
SDD, subantimicrobial doses of doxycycline. [a]P<0.05, indicates a significant difference between groups.			

A significant percentage of patients, both in the study group and in the control group, showed radiological signs of odontogenic sinusitis, totaling 29 patients (56.86%).

In terms of patient compliance, 34 subjects were initially included in the SDD group, but 3 of them (8.82%) did not complete the regimen with subantimicrobial doses of doxycycline. All subjects included in the control group followed the study methodology.

Bleeding index. Following the evaluation of the bleeding index in the SDD group, we observed a significant decrease at the end of the therapy with subantimicrobial doses of doxycycline (T1), a decrease that continued at the 3 (T2) and 9 month (T3) assessments after the completion of the SDD therapy ($P<0.001$). For the control group, we noted significant decreases for the bleeding index after 3 months (T1); however, this followed an increasing trend at 6 (T2) and 12 months from baseline (T3), approaching the initial values (Fig. II.18).

Probing depth. The determination of probing depth at 3 months from baseline (T1) revealed lower values in patients in the SDD group, even though it did not reach the statistical significance threshold; these values continued to decrease throughout the study, the difference being significant at the 6 (T2) and 12 month (T3) assessments from baseline ($P<0.05$). Despite the average value of the probing depth being lower than the baseline at 3 months for the control group, this difference was not statistically significant. Moreover, the values increased at T2 and T3 evaluations (Fig. II.19).

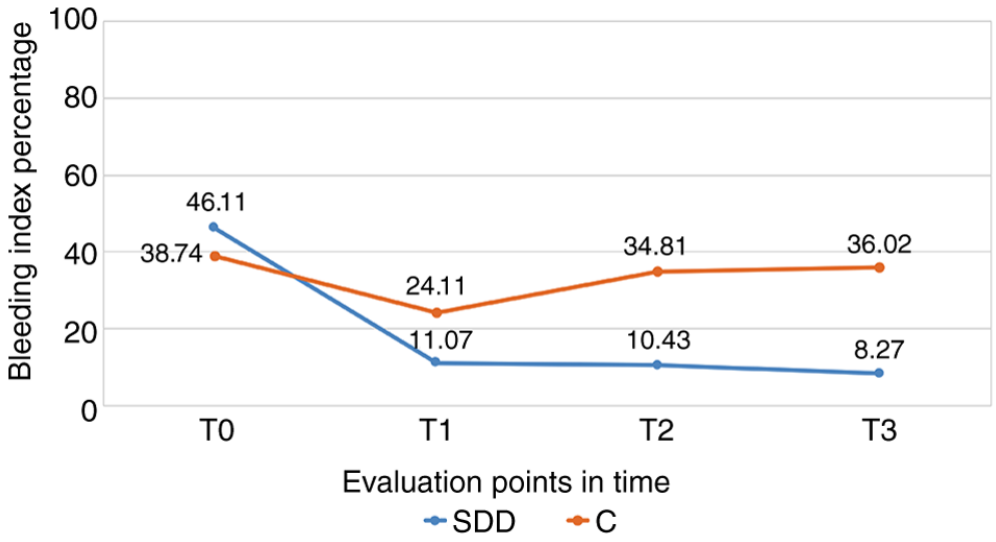

Figure II.18. Bleeding index variation at evaluation time points: T0, baseline evaluation (before treatment); T1, evaluation after SDD (3 months from baseline); T2, evaluation at three months after SDD (6 months from baseline); T3, evaluation at nine months after SDD (12 months from baseline). SDD therapy exerted a significant decrease in bleeding, maintained through the study period (blue). In the control group (red), the bleeding index was lower immediately after standard therapy but there was a strong increase at 6 and 12 months from baseline, approaching the initial values. SDD, doxycycline at subantimicrobial doses.

Loss of periodontal clinical attachment. When assessing the loss of periodontal clinical attachment after the completion of SDD therapy (T1), the value was lower, but did not reach a clinical significance threshold. Importantly, in the 6 (T2) and 12 month (T3) evaluations, we noted a decreased tendency of these values in the SDD group. In the control group, consisting of patients who only followed conventional therapy, CAL decreased significantly when assessed 3 months (T1) after the initial moment. Nevertheless, similarly to the other periodontal parameters, it showed an upward trend in the evaluations from 6 (T2) and 12 months (T3), the last of them revealing a value even higher than the initial one (Fig. II.20).

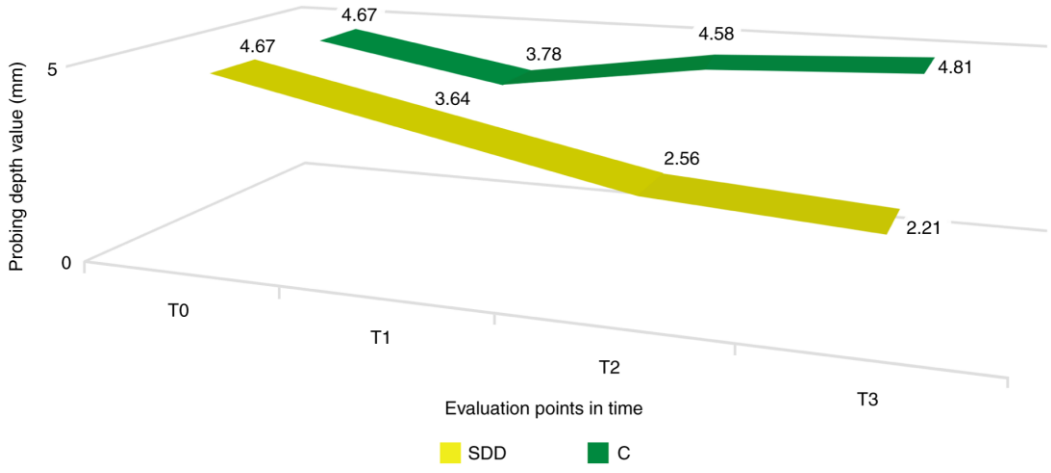

Figure II.19. Probing depth variation at the evaluation time points: T0, baseline evaluation (before treatment); T1, evaluation after SDD (3 months from baseline); T2, evaluation at three months after SDD (6 months from baseline); T3, evaluation at nine months after SDD (12 months from baseline). In the SDD group the probing depth decreased throughout the study (yellow). In the control group the values increased at 6 and 12 months (green). SDD, doxycycline in subantimicrobial doses.

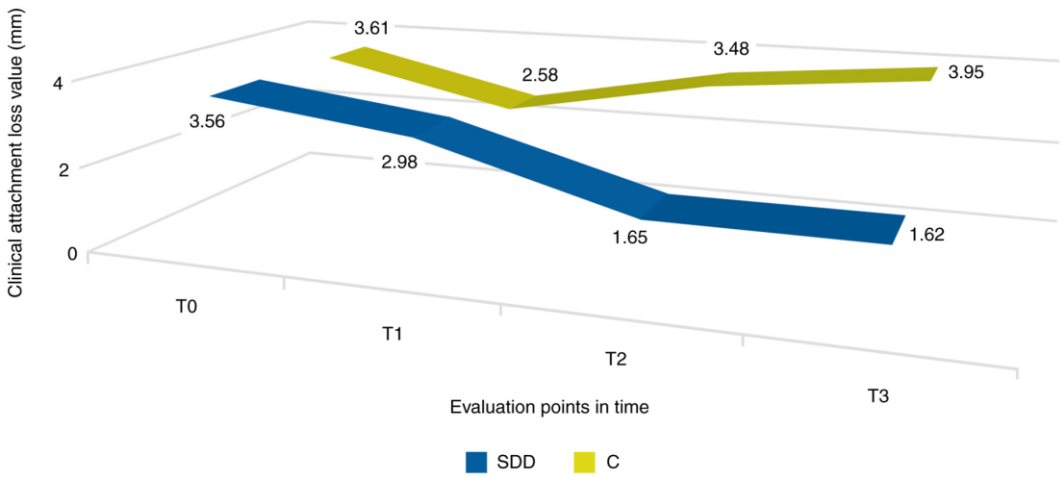

Figure II.20. Clinical attachment loss (CAL) variation at evaluation time points: T0, baseline evaluation (before treatment); T1, evaluation after SDD (3 months from baseline); T2, evaluation at three months after SDD (6 months from baseline); T3, evaluation at nine months after SDD (12 months from baseline). In the SDD group in the 6 and 12 month evaluations we noted a deceasing tendency for the CAL values (blue). In the control group, CAL showed an upward trend in the evaluations at 6 and 12 months (yellow). SDD, doxycycline in subantimicrobial doses.

At the beginning, we did not note significant differences between groups for any of the analyzed periodontal parameters. At the end of the SDD therapy (T1), only the bleeding index showed significantly lower values for the SDD group compared to the control group (P=0.0311), but 3 (T2) and 9 (T3) months after the completion of the SDD therapy, we observed significantly lower values for all the examined periodontal parameters.

Regarding the level of glycated hemoglobin, at T1we noted significant decreases for both study groups. The differences between the SDD group and the control group were significant when compared at the T2 and T3 assessments (P=0.0025 and 0.0002, respectively).

For the group of patients with diabetes who underwent subantimicrobial doses of doxycycline therapy HbA1c, it continued to decrease, while for the group of patients who only followed conventional therapy, these values began to increase, approaching the baseline values (Table II.26).

Table II.26. Mean values of glycated hemoglobin in the study groups.

	SDD group				Control group				V0	V1	V2	V3
	T0	T1	T2	T3	T0	T1	T2	T3				
HbA1c (%)	8.8±1.8	7.2±1.6[a]	7.1±1.7[a]	6.8±1.5[a]	8.9±1.9	7.1±1.5[a]	8.3±1.6	8.7±1.8	0.852	0.741	0.0025[b]	0.000[b]

SDD, subantimicrobial doses of doxycycline; HbA1c, glycated hemoglobin. T0, baseline evaluation (before treatment); T1, evaluation after SDD (3 months from baseline); T2, evaluation at three months after SDD (6 months from baseline); T3, evaluation at nine months after SDD (12 months from baseline); V0, P-value between groups at baseline; V1, P-value between groups at T1; V2, P-value between groups at T2. Values are expressed as mean value ± standard deviation (SD). [a]P<0.05, compared with the T0 value , [b]P<0.05, SDD group compared to Control group.

Discussion

Comparisons between patients with diabetes and those in the control group led to the observation that diabetes constitutes a risk factor for periodontal disease in general and for endo-periodontal lesions in particular. Oral disorders, such as periodontal disease, as well as diabetes, are multifactorial diseases (Liccardo et al., 2019). More obviously, diabetic patients are susceptible to various forms of periodontal disease, a particular importance being given to the diabetes-periodontal disease relationship, with the identification of patients who are more prone to these types of oral disorders (Hegde & Awan, 2019).

Diabetes is known to decrease the host resistance to infections and diminish wound healing. Insulin is required for glucose uptake into cells and to provide an energy source for amino acid amelioration in protein synthesis, as well as for preventing lipolysis of adipose tissue. If insulin administration is not sufficient, then the basic cell functions of the body will be disrupted. Signs of host defense against microbes are well known: impaired polymorphonuclear leukocytes (PMN) cell function with adhesion abnormalities, chemotaxis, phagocytosis, and intercellular destruction. Type 2 DM is associated with a series of microvascular complications that most commonly affect the eyes and kidneys, and histopathological studies have shown internal ear nerve and vessels damage in subjects with diabetes (Graves et al., 2020).

An important complication related to the poor glycemic control, with great effect on the quality of life, bacterial, fungal or viral infections, are common in patients with diabetes and can affect the skin and soft tissue structure of the ear and nose. Both hypoglycemia and hyperglycemia have been associated with internal ear dysfunction, and hearing can fluctuate with the level of glycemic control. The relationship between diabetes mellitus, sensory hearing loss and vestibular dysfunction is known, and histopathological changes of the temporal bone have been clearly documented (Gazzaz et al., 2011).

The duration of diabetes is an important factor that causes the occurrence of microvascular complications of diabetes (ADA, 2014). It seems that the longer duration of diabetes mellitus predisposes to the development of deafness in many studies; however, a mild degree of hearing impairment has been detected in many children with diabetes lasting more than four years. Such an observation was unusual and may be explained by poor glycemic control. Elamin *et al* (2005) confirmed the relationship of loss hearing in children and adolescents with type 1 diabetes mellitus at medium and high frequencies.

In diabetic patients with endo-periodontal lesions, periodontal therapy may have beneficial effects on glycemic control (Hegde & Awan, 2019). This may be especially true for patients with relatively poorly controlled diabetes and more advanced periodontal destruction prior to treatment.

An understanding of the effects of other infections would be helpful in delimiting the mechanisms by which periodontal infection influences blood sugar. Acute bacterial and viral infections have been shown to increase insulin resistance and worsen metabolic control. This occurs in individuals with or without diabetes. Systemic infections increase tissue resistance to insulin, preventing the entry of glucose into the target cells, leading to an increase in blood sugar and requiring an increase in insulin production to maintain a normoglycemic state (Hegde & Awan, 2019).

A systematic review examining the etiology of odontogenic sinusitis in a group of 674 patients showed that an iatrogenic etiology accounted for 65.7% of cases, apical periodontal pathology accounted for 25.1% of cases, and marginal periodontitis accounted for 8.3% of cases (Lechien et a., 2014). This study further demonstrated that the most frequently affected maxillary teeth, in order of frequency, were the first molar (35.6%), the second molar (22%), the third molar (17.4%) and the second premolar (14.4%) (data not shown). Thus, there is an increased risk in patients with combined endo-periodontal lesions, especially if these lesions

also affect the furcation area. In the present study, we noted a significant percentage of patients with endo-periodontal lesions who had radiological signs of odontogenic sinusitis, a diagnosis subsequently confirmed by CBCT examinations. In the context of the presence of diabetes, patients with endo-periodontal lesions are at high risk of local and loco-regional complications, including odontogenic sinusitis; this risk is amplified in cases of poor glycemic control. Therefore, modulation of the inflammatory response makes a significant contribution in mitigating these risks.

Several types of meta-analyses have confirmed that effective periodontal therapy may result in reduced glycated hemoglobin A1c (HbA1c). The first reported was performed on 10 interventional studies, with a combined population of 456 patients; the authors identified a weighted average HbA1c reduction of 0.66% as a result of periodontal therapy (although this failed to reach statistical significance) (Janket et al., 2005). In 2010, a meta-analysis of 5 studies involving 371 patients also reported a significant weighted average HbA1c reduction of 0.40% at a 3-9 months follow-up period (Teeuw et al., 2010).

The Cochrane collaboration reported studies that investigated the relationship between periodontal treatment and glycemic control in people with diabetes. Three studies were included in this meta-analysis that reported a significant reduction of 0.40% HbA1c at 3-4 months after conventional periodontal therapy (Simpson et al., 2010). The findings of these meta-analyses are supported by a population study of over 5,000 people with diabetes, reporting that patients who had at least one periodontal access surgery session had HbA1c levels that were 0.25% less than patients who had not undergone periodontal surgery (Spangler et al., 2010).

Taken together, the evidence supports the idea that improvements in metabolic control can be anticipated following the effective treatment of periodontitis. The mechanisms by which this happens is not yet clear, but probably is due to reduced systemic inflammation (e.g., low serum concentrations of mediators such as TNF-α and IL-6), after treatment and resolution of periodontal inflammation (ADA, 2014). These observations are important as reductions in HbA1c are associated with a reduced risk of diabetes complications. For example, it was found that each 1% reduction in HbA1c is associated with a 21% risk reduction for any diabetes-related complication, 21% for diabetes-related deaths, 14% for myocardial infarction, and 37% for microvascular complications (Stratton et al., 2000).

Diabetes affects many functions of the immune system and is associated with delayed healing and compromised immune responses. Diabetes-induced changes in immune cell function produce an inflammatory immune cell phenotype (stimulation of pro-inflammatory cytokines from monocytes/polymorphonuclear leukocytes and inhibition of macrophage growth factors). This predisposes to chronic inflammation, progressive tissue breakdown and diminished tissue repair capacity (Nayak et al., 2013).

Doxycycline and other tetracycline analogues have been shown to reduce tissue protein glycation in animals with streptozotocin-induced diabetes without apparent changes in serum glucose levels. Therefore, we hypothesized that doxycycline may be useful in the treatment of patients with diabetes by reducing protein glycation. The hypothesis of this study showed that SDD could play a role in reducing protein glycation in humans.

The implications of this study have far-reaching potential if the results are confirmed in larger and long-term studies. Firstly, SDD has already been approved for the adjuvant treatment of periodontitis. As patients with diabetes have a high risk of periodontitis, increased use of this type of therapy in the population will improve the results of periodontal treatment and may lead to improvements in diabetes outcomes. Secondly, we did not observe any increased incidence of adverse events in patients with type 2 diabetes who had SDD for three months (data not shown). Thirdly, subjects took stable doses of oral hypoglycemic

agents and/or insulin and no adverse events were observed, indicating an apparent lack of adverse drug interactions between SDD and these agents.

As described in a larger number of studies on SDD in non-diabetic populations, no serious adverse events were observed in this study and SDD appeared to be well tolerated (Payne et al., 2011). Therefore, the use of SDD appears to be safe and effective for the treatment of endo-periodontal lesions in subjects with type 2 diabetes. However, these data should be interpreted with caution, given the small sample size. Clearly, larger studies are needed in subjects with type 2 diabetes to confirm whether this treatment is safe and effective for the treatment of endo-periodontal lesions in patients with type 2 diabetes and to test whether SDD is an effective adjuvant drug for the treatment of diabetes. It also remains to be determined whether long-term administration of SDD is safe and effective in reducing the complications of diabetes. However, based on these pilot data, longitudinal studies appear to be warranted.

Conclusions

Therefore, subantimicrobial doses of doxycycline generated favorable results for the evaluated periodontal parameters (bleeding index, probing depth and clinical periodontal attachment loss) and, unlike conventional therapy, these results were maintained over time.

Moreover, we demonstrated that adjunctive therapy with SDD had a clear contribution to improving glycemic control in patients with diabetes and endo-periodontal lesions, an improvement manifested by significantly reduced glycated hemoglobin levels throughout the study (12 months). This fact has far-reaching effects in the sphere of loco-regional complications as well and the risk of odontogenic sinusitis can be significantly reduced.

In addition, subantimicrobial dose therapy of doxycycline was well tolerated, with no adverse effects, which contributes to its recommendation in the therapeutic management of patients with diabetes mellitus and endo-periodontal lesions.

II.3.4 Desensitizing agents in the treatment of dentin hypersensitivity

State of the art in dentin hypersensitivity therapy

According to the most widely accepted definition, dentin hypersensitivity (DH) represents a short, sharp pain which results as a response of exposing dentin to various stimuli (thermal, tactile, osmotic or chemical) and cannot be ascribed to any other form of dental defect or pathology (Dowell & Addy, 1983).

The exposure of cervical dentin is a common condition for the onset of DH. Dental trauma, gingival recession, periodontal treatments, aggressive tooth brushing can lead to dentin exposure. Frequent exposure to acidic beverages might determine erosions with high demineralization of exposed dentin and enlargement of the dentinal tubules (Stoleriu et al., 2014).

The commercially available products are not always effective in releasing dental hypersensitivity, especially on short term. Therefore, new products have been developed to address this disturbing pathology by mechanisms specifically designed to counteract the DH phenomena.

Two therapeutic strategies have been used to reduce or to eliminate DH: blocking neural transmission at the pulpal tissues by chemically depolarizing the nerve synapse and occlusion of the dentin tubules (Davari et al., 2013). The first mechanism is still controversial, because of the long diffusion distance and outward flow of the dentinal fluid.

On the other hand, the occlusion of dentinal tubules is well documented and might be obtained physiologically, by mineral crystals formation in the intratubular area due to dentinal fluids and saliva (Yoshima et al., 1990) or therapeutically by application of chemical

agents. The occlusion of dentin tubules influences the hypersensitivity in two different ways: by blocking the tubules which decrease the dentinal fluid flow and by creating a barrier against the stimuli from the oral cavity.

A large variety of active ingredients were included in the at-home or professional products used for dentin desensitizing: fluorides, strontium salts, arginine and calcium carbonate, oxalates, calcium phosphosilicates, nanoparticles like bioreactive glass. Various mechanisms are involved with controversial results. Strontium salts are absorbed by dentin and form strontium apatite, while arginine combined with calcium carbonate can occlude the dentin tubules with calcium phosphate and stannous fluoride produces an acid-resistant precipitate on dentin surface (Saeki et al., 2016).

Phosphosilicates precipitate onto dentin collagen and create deposits located on the dentin surface and in the dentin tubules (Joshi et al., 2013) and oxalates form calcium crystals within the dentin tubules and might block the dentinal fluid flow. Fluoride which is the most common component of toothpastes might increase the mineralization of hydroxyapatite (Naumova et al., 2010).

This research direction has been materialized by publishing the following paper:

Simona Stoleriu, Galina Pancu, Angela Ghiorghe, Dorina Cerasella Sincar, Sorina Solomon, Sorin Andrian, Gianina Iovan. Evaluation of dentinal changes following application of three different deensitizing agents. REV. CHIM (Bucharest) 2017; 68(7):1573-1577
http://www.revistadechimie.ro/pdf/34%20STOLERIU%207%2017.pdf

II.3.4.1 ***Evaluation of dentinal changes following application of three different desensitizing agents***

Aim of the study

Numerous studies have evaluated various products and therapeutical protocols for desensitizing teeth. Still there is no agreement about the most effective products and application method. A recent systematic review concluded that there is no agent that can provide complete relief from dentinal hypersensitivity (Da Rosa et al., 2013).

Considering these controversial results, the aims of our study were to analyse the morphological changes of dentin and to assess the degree of tubules occlusion when 3 commercial desensitizing toothpastes having different active ingredients were used.

Materials and method

Fifty caries-free human teeth extracted for orthodontic reasons were used for this study. The teeth were obtained from the Department of Maxillofacial Surgery of the Faculty of Dental Medicine. All the teeth were stored in 0.9% NaCl until the start of the experiment. Thirty dentin discs having a thickness of 3 mm were obtained by cutting the teeth in the mesial-distal direction perpendicular to the long axis of the teeth, using diamond discs (Komet Dental, Brasseler GmbH&Co, Germany) at low speed under cooling water.

The discs were randomly and equally assigned to three groups (groups 1-3). All the discs were submersed in citric acid for 30 s to open the dentin tubules. Then the discs were cut in two halves. In each group 10 halves were kept in 0.9% NaCl solution (control groups 1-3) and the other 10 halves were exposed to the action of one of the tested desensitizing toothpastes (study groups 1-3). The dentin samples were placed in the machine designed to simulate the forward and backward movements during tooth brushing with amplitude of 30 mm (15 mm in each direction) and frequency of 60 cycles/min.

Toothbrushes having medium hardness of the bristles were used to simulate the regular

tooth brushing (Colgate Classic Deep Clean). The load applied on each toothbrush was 250 g. The calculated mean number of toothbrush cycles during a tooth brushing of 2 minutes was 20. In order to simulated the toothbrush for 2 min, twice a day, 30 days, all the samples in the study groups were brushed continuously for 2 h, which is in accordance with the protocols used in previous studies (Arnold et al., 2015).

Table II.27. The ingredients of the toothpastes used in the study

Toothpaste	Active	Inactive ingredients	Producer	Batch no
Sensodyne Repair and protect	Stannous fluoride 0.454% (0.15% w/w fluoride ion)	glycerin, PEG-8, hydrated silica, pentasodium triphosphate, sodium lauryl sulfate, flavor, titanium dioxide, polyacrylic acid, cocamidopropyl betaine, sodium saccharin	Glaxo Smith Kline	
Sensodyne Rapid	Strontium acetate hemihydrate 8.0% w/w, sodium fluoride 0.23% w/w (0.104% w/w fluoride ion).	Aqua, Sorbitol, Hydrated Silica, Glycerin, Sodium Methyl Cocoyl Taurate, Xanthan Gum, Titanium Dioxide, Aroma, Sodium Saccharin, Sodium Propylparaben, Sodium Methylparaben, Limonene	Glaxo Smith Kline	
Colgate Sensitive Pro Relief	Arginine 8%	Calcium Carbonate, Aqua, Sorbitol, Bicarbonate, Sodium Lauryl Sulfate, Sodium Monofluorophosphate (1450 ppm F), Aroma, Cellulose Gum, Sodium Bicarbonate, Tetrasodium Pyrophosphate, Titanium Dioxide, Benzyl Alcohol, Sodium Saccharin, Xanthan Gum, Limonene.	Colgate	

Three commercial desensitizing toothpastes were chosen to be applied on dentin surface: Sensodyne Repair and Protect (Glaxo Smith Kline) (Group 1), Sensodyne Rapid Release (Glaxo Smith Kline) (Group 2) and Colgate Sensitive Pro Relief (Colgate) (Group 3). The active and inactive ingredients of the toothpastes are summarized in Table II.27. Slurries were prepared by mixing water and toothpaste (1:2 by volume) and dispensed on the samples surface with a frequency of 1mL\min. SEM evaluation of the samples and assessment of dentin tubules occlusion.

All the prepared samples in control and study groups were evaluated using scanning electron microscope VEGA II LSH (Tescan Czech Republic). Ten standardized images of each dentin surface were obtained at a magnification of 1000X. Two different examiners, trained before the experiment and blinded to the materials used, evaluated the morphology of dentin samples and scored the level of tubule occlusion on a scale from 1–5 according to the tubule occlusion classification scoring system (West, Davies): 1-occluded (100% of tubules occluded); 2-mostly occluded (50–<100% of tubules occluded); 3-partially occluded (25–<50% of tubules occluded); 4-mostly unoccluded (<25% of tubules occluded); 5-unoccluded (0%, no tubule occlusion). The two examiners confronted the score given for each image and the final score resulted as a common decision of both examiners. For each sample the mean score of ten assessments was used for analysis.

Results

SEM micrographs of dentin samples in all control groups showed large opening of dentin tubules (Figure II.21.a-c). In the Study Group 1, some of the dentin tubules seemed to be totally or partially occluded, whilst some remained opened (Figure II.21d).

Evaluation of dentin surface in Study Group 2 showed the dentin surface covered by particle deposition and very few opened tubules (Figure II.21e).

The samples in the Study Group 3 showed most of the dentin tubules occluded and

large deposition of mineral particles on the intertubular dentin (Figure II.21f). The particle deposition was obvious in Study Groups 2 and 3 when compared to Study Group 1.

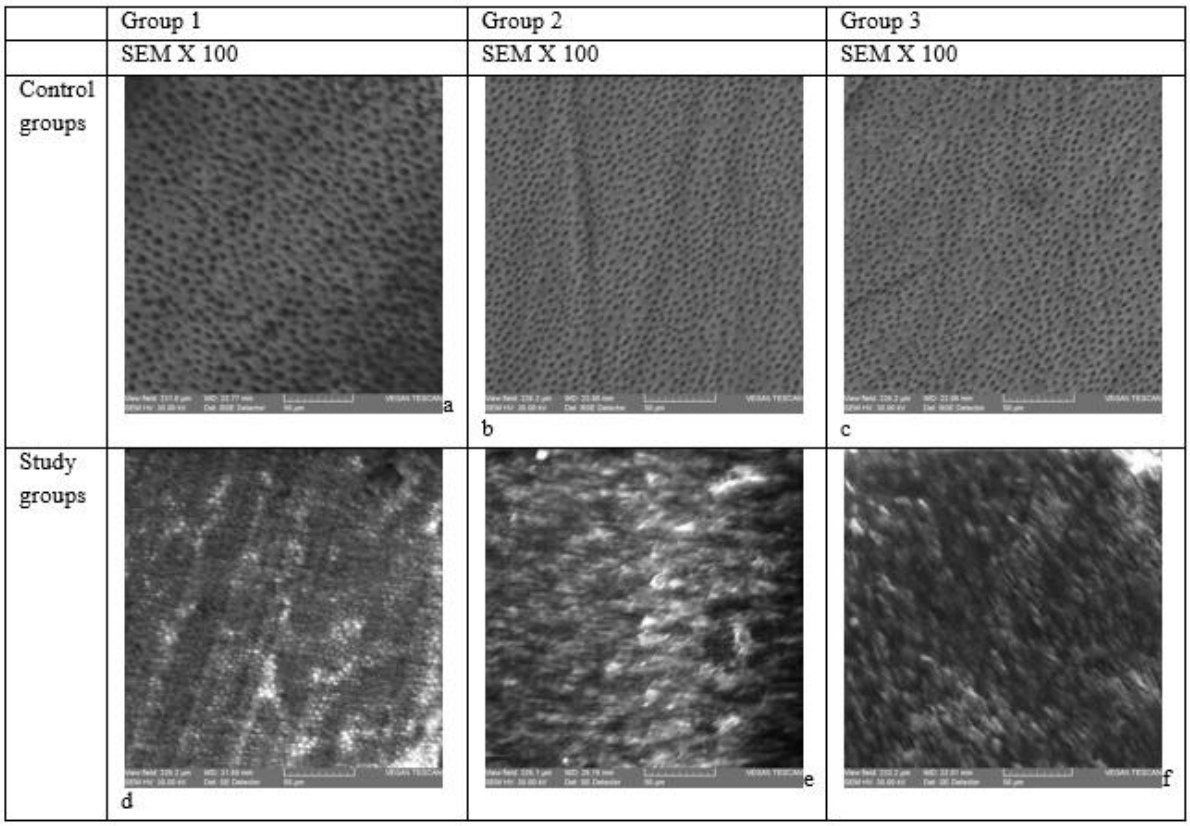

Figure II.121. SEM micrographs of dentin samples in control groups 1-3 (a-c) and study groups 1-3 (d-e)

The mean scores of tubule occlusion for the control and study groups are presented in Table II.28. In all the study groups the score values were lower when compared to control, as a result of increased tubule occlusion. Group 3 presented the lowest values of the score and Group 1 the highest.

Table II.28. Mean scores of tubule occlusion for control and study groups

	Group 1	Group 2	Group 3
Control	5.0	5.00	5.00
Study	3.25	2,67	2.12

The values distribution in groups was evaluated using Shapiro-Wilk normality test. A p value lower than the chosen alpha level of 0.05 rejected the null hypothesis of normal distribution of values in group.

Due to the fact that in Study Group 1 the variable was not normally distributed (Table II.29), non-parametrical Mann-Whitney U test was chosen to compare the results in the groups.

Table II.29. Results of normality Shapiro-Wilk Test

	Kolmogorov-Smirnov[a]			**Shapiro-Wilk**		
	Statistic	Df.	Sig.	Statistic	Df.	Sig.
Group 1	.268	10	.040	.829	10.	**.033**
Group 2	.224	10	.168	.911	10	.287
Group 3	.240	10	.108	.858	10	.073
a.Lilliefors Signigicance Correction; b. Control is constant, it has been omitted						

Significantly statistical differences were obtained when compared the tubule occlusion scores in study groups and control groups. Also, significantly statistical differences were obtained when compared the values between the study groups (Table II.30).

Table II.30. Results of Mann-Whitney U statistical test

	z	Asymp. Sig. (2-tailed)
Control-Group 1 study	-4.058	0.000
Control-Group 2 study	-4.065	0.000
Control-Group 3 study	-4.065	0.000
Group 1-Group 2 study	-3.817	0.000
Group 2-Group 3 study	-3.823	0.000
Group 1-Group 3 study	-3.817	0.000

Discussion

The in-vitro assessment of dentinal blockage by dentin disc model has been widely used for evaluating the efficiency of hypersensitivity treatment (Ansari et al., 2015).

Our results suggested that all the tested products determined a significant occlusion of the dentinal tubules. The ascending sequence of the mean scores of tubule occlusion was: Colgate Sensitive Pro-Relief; Sensodyne Rapid; Sensodyne Repair and Protect with statistical significant differences among all groups. Our results were consistent with several previous studies and inconsistent with others, each of them testing at least one of the dentifrices or one of their active ingredient.

Arnold et all compared the number of dentin tubules after using 6 toothpastes including Sensodyne Repair, Sensodyne Rapid and Elmex Sensitive Professional which had similar active ingredients as Colgate Sensitive Pro-Relief (Pro-Argin and calcium carbonate) (Arnold et al., 2015). Significant differences of the tubule occlusion after brushing with the toothpaste comparing with the specimens brushed with artificial saliva were found only for Sensodyne Rapid. When comparing to the positive control represented by specimens brushed with conventional toothpaste, the differences were significant for both Sensodyne Repair and Sensodyne Rapid but not for Elmex Sensitive Professional.

The different technique of evaluation might explain the controversial results. The SEM examination combined with EDS analysis demonstrated irregular coverage of dentin surface by silica, which is an abrasive used in dentifrice and not an active ingredient. A scattered thin layer of silicon covered the dentin surface in case of Elmex Sensodyne Professional but not in the case of Sensodyne Repair. In our study, an irregular smear layer was observed in all the study groups, the deposition being more significant in case of Sensodyne Rapid and Colgate Sensitive Pro-Relief, however the composition of these remnants was not analysed in our study.

However, the better results achieved with Colgate Sensitive Pro-Relief in our study are supported by other researchers. Most of the studies demonstrated a good occluding effect of Pro-Argin products on open dentin tubules (Joshi et al., 2013; Parkinson & Willson, 2011; Patel et al., 2011) which also explain the results of the clinical studies showing an instant relief of dentin hypersensitivity (Ayad et al., 2009). The two active ingredients, arginine and calcium, are found naturally in saliva and might have a role in natural occlusion of the dentinal tubules and formation of the protective layer on the dentin surface.

Chen et al. (2015) found that applying Colgate Sensitive Pro-Relief on dentin samples resulted in significantly more tubule occlusion when compared to Novamin and salin. The mean score of tubule occlusion was 2.45, which is close to our result. They concluded that even after short term application the tubules of the samples were occluded by crystal-like deposition.

Our results are also in agreement with previous in vitro studies (Petrou et al., 2009; Lavender et al., 2010), demonstrating that arginine combined with calcium carbonate occlude dentine tubules. Mahale et al. (2015) found that after 7 and 14 days of applying Arginine, clear tubule occlusion was noticed. This was thought to be caused by deposition of the calcium carbonate and arginine agglomeration within the tubules.

These findings explain the results of clinical trials showing the efficacy of arginine - calcium carbonate in relieving DH (Kapferer et al., 2013). The mechanism of action was explained by Kleinberg who suggested that the combination of arginine and calcium carbonate forms a positively charged complex which readily binds to the negative charged dentin surface and within the dentin tubules. In addition, the alkaline pH of arginine-calcium carbonate facilitates the deposition of calcium and phosphate from saliva/and or dentinal fluid to form plugs that seal the patent tubules.

In vitro studies suggested that the deposit converts to calcium phospahate (Lavender et al., 2010). The remineralisation process consists in the precipitation of calcium and phosphate as insoluble Calcium Phosphate which, when present in the saliva, is brought to the demineralised enamel in incipient defects resulted from surface demineralization.

As regarding Sensodyne Rapid, strontium can be found naturally in human enamel and dentin as a trace element; strontium is a re-mineralizing agent, and gets incorporated into the mineral phase of enamel by replacing calcium. The occlusion of the tubules can be explained by the affinity for dentin and possibility for adsorption into or onto the organic tissues. In vitro studies have shown that strontium acetate treatments form small crystalline deposition on the dentin surface (Addy & West, 2013).

The data about the efficiency of stannous fluoride, which is the main active ingredient of Sensodyne Repair and Protect are controversial (He et al., 2014). In Arnold's study, no dentinal occlusion of the cross-sections of dentin could be observed after the treatment, however the examination of the surface revealed occluded tubules which is in accordance with our results (Arnold et al., 2015).

Conclusions

In our study all the tested dentifrices had a significant effect on dentin in terms of occluded tubules. Whether the blockage of the tubules at the dentin surface is a result of the active ingredients or also a consequence of the retention of the abrasive agents of toothpastes or smear layer formation is still a matter of debate. Both active ingredients and passive mechanisms may contribute to the desensitizing effect on short term.

However, the persistence of the desensitizing effect in the oral environment on long term is probably dependent on several characteristics such as resistance to corrosion, abrasion and and formation of occluding tags into the tubules. Further experiments to investigate these characteristics are necessary for the assessment of long-term effects of theses desensitizing toothpastes.

All the three desensitizing toothpastes demonstrated significant effects on dentinal tubule occlusion. The tooth paste containing arginine and calcium carbonate as active ingredients showed the highest degree of tubule occlusion, followed by the dentifrice containing strontium acetate and sodium fluoride.

Further research is required to analyse the influence of the application protocol on the results and to evaluate the durability of the effects under chemical and mechanical challenges.

II.4 The influence of systemic therapies on the periodontal status in patients with affected general status

State of the art in the potential influence of systemic therapies on periodontal status

Frequently, the patients with periodontal disease and chronic systemic diseases also undergo systemic treatment for the precise disease. The various kinds of drugs can exert a positive or, contrary, a negative influence on the outcome of the periodontal treatment.

Positive examples include THS in post-menopausal female patients with osteoporosis, DMARDS in rheumatoid arthritis or usage of statins in CVD patients.

Estradiol (a steroid hormone derived from cholesterol necessary for the maintenance of fertility and secondary sexual characteristics in women) inhibits the expression of certain cytokines. After menopause, its rate decreases and there is a lifting of inhibition and therefore an increase in the release of certain cytokines at the local level as at the systemic level (Bouchard, 2015).

Oestrogen deficiency would promote periodontitis either by causing the increased expression of proinflammatory cytokines or by reducing the bone mass of the maxilla. In animal models, oestrogen deficiency exacerbates the severity of periodontitis. Ovariectomized rats have a higher expression of IL-6, RANKL, of osteoprotegerin (OPG) in periodontal tissue, suggesting the impact of the hormone oestrogen on inflammatory bone resorption (Wang & McCauley, 2013).

The studies devoted to such aspects also demonstrated that THS reduces the loss of mandibular alveolar bone tissue during the menopause and, in some cases, even promotes an increased bone mineral density. Another longitudinal study developed on a group of 41 women at postmenopause, who had received THS for periods between one to two years, evidenced a positive effect of THS as to the loss of mandibular bone mass (Tarkkila et al., 2008).

The positive results registered by hormonal therapy applied upon the alveolar bone mass support the conclusions of other observational studies on edentations and periodontal diseases, consolidating the idea that TH increases tooth attachment and improves dental health.

Even if the high levels of ovarian hormones during pregnancy and the utilization of oral contraceptives may increase gingival inflammation and also the gingival exudate, menopause – the absence of ovarian steroides – has been related to the alteration of the general gingival health condition, a situation in which the therapy of hormonal substitution may apparently support this tendency.

Rheumatoid arthritis and periodontal disease share similar inflammatory pathways and environmental mechanisms. This potential association has generated new ideas about possible links between these two common conditions. Although early mechanisms that have as a result impaired immune tolerance and progression to AR signs and symptoms are not known, the inflammatory cascade plays a key role in all stages of the pathogenesis of this disease, from initiation of autoimmunity to articular localization and destruction of joints and bones (Schmickler et al., 2017).

Treatment modalities in patients with RA and periodontitis can include medications, efforts to reduce joint stress, physical therapy and surgery. Non-steroidal anti-inflammatory agents (NSAIDs) and so called anti-rheumatic disease-modifying drugs (DMARDs) are commonly used to treat RA. The term DMARDs is used to name a group of drugs that are generally unrelated, but differ from NSAIDs that reduce inflammation but do not treat RA and steroids that reduce the immune and inflammatory response but do not slow the progression of the disease. In other words, while NSAIDs and steroids are used to control RA symptoms, only DMARDS influences the progression of the disease (Smolen et al., 2007).

DMARDs determine the reduction of the level of CRP and FR markers, the rate of erythrocyte sedimentation, and cartilage and bone damage. In the treatment of rheumatoid arthritis, the therapy with biological DMARDs are often prescribed in combination with a conventional agent in those patients that presented a limited response to conventional anti-rheumatic disease-modifying therapy. Biological DMARDs include a number of anti-cytokine agents that block the activity of specific cytokines and are usually monoclonal antibodies that bind to the target cytokine.

Some research has shown that patients with autoimmune disorders (including AR) exhibit much more severe periodontal inflammation than patients who do not suffer from autoimmune disease, anti-TNF-α therapy decreasing inflammation in periodontal tissues (Mayer et al., 2013).

We also conducted a study which proposed an analysis of the local inflammatory status by evaluating Quigley Hein indices, papillary bleeding (PBI) index and gingival index Lõe and Silness, accompanied by a detailed assessment of systemic status in patients with rheumatoid arthritis and *synthetic + biologic DMARDS therapy.*

The treatment for RA was mainly based on the following: most commonly Leflunomide (46.4%) and Rituximab (44.5%); Methotrexate was also noted to be administrated with a frequency of 23.6%; Adalimumab and Tocilizumab were also reported to be administrated with a frequency of 18.2% and 15.5%, respectively. The lowest mean VSH level is seen in Hydroxychloroquine + Rituximab and Metotrexate + Adalimumab treated patients, and the highest is seen in subjects receiving Hydroxychloroquine + Tocilizumab. Regarding the classical inflammatory marker, the lowest average CRP score is seen in patients treated with Methotrexate + Adalimumab, and the highest in subjects treated with Hydroxychloroquine + Tocilizumab. Measurement of oral markers revealed the lowest median Quigley Hein score in patients treated with Sulfasalazine + Rituximab, while the highest values were recorded in Leflunomide + Etanercept therapy.

The lowest average GI index level was seen in patients treated with Methotrexate + Adalimumab and Leflunomide + Adalimumab, and the largest was found in patients treated with Leflunomide + Rituximab and Metotrexate + Rituximab. The lowest average PBI level was observed in Leflunomide + Etanercept treated patients, and the highest in patients treated with Leflunomide + Rituximab.

The biological reason for using DMARDS treatment as a modulator of periodontitis expression in animals was confirmed by studies that showed that mice with TNF-α deficient p55 receptor developed less severe periodontal inflammation (reduced bone loss and low inflammatory response) in response to inoculation with *A. actinomycetemcomitans* (Garlet et al., 2007).

Using the same experimental periodontitis model induced by *A. actinomycetemcomitans*, the researchers found that antigen-induced arthritis exacerbated alveolar bone loss, while anti-TNF-α therapies improved the development of periodontitis (Corrêa et al. 2017).

In our study, the combination of methotrexate and adalimumab recorded the lowest VSH and CRP values, consistent with multiple studies demonstrating the systemic anti-inflammatory effect of methotrexate in particular (Ramiro et al., 2017; Orr et al., 2018). Leflunomide and adalimumab associations, as well as leflunomide with etanercept, recorded the most marked decreases in both rheumatic and oral health indices, similar to those of which identified that patients with rheumatoid arthritis that received anti-TNF-α treatment experienced statistically significant improvements of the probing depth, probing bleeding and gingival inflammation compared to patients that did not receive anti-TNF-α therapy after non-surgical periodontal treatment.

The combination of leflunomide with rituximab or methotrexate with rituximab was

associated with the highest values of GI and PBI indices, coinciding with similar literature studies (Ziebolz et al., 2018).

However, it is important to note that, in contrast, in patients receiving biological DMARDs treatment, the average level of oral health indices was significantly lower. Thus, we can note the positive influence that this class of drugs, especially those that act on TNF-α, generates on local status (Nedeff et al., 2015).

In order to maintain oral health, patients with RA are encouraged to achieve proper oral hygiene. Consultation of the periodontist is necessary to determine the course of treatment. Reducing the oral contribution to the overall inflammatory burden due to the favourable outcome of periodontal treatment is an important desideratum. Maintaining the full health of RA patients should be a collaborative effort. This dentist-rheumatologist partnership will definitely influence the oral and global health of these patients.

Interventions to improve oral pathology may have direct and indirect systemic benefits. Considerations include the patient's ability to maintain adequate oral hygiene, xerostomia and the related complications, patient susceptibility to infections, haemostasis alterations, and drug actions and interactions.

This research direction has been materialized by publishing the following papers:

1.Nicolaiciuc O, Mihai C, Sufaru IG, Martu I, Solomon SM, Tatarciuc D, Budacu C, Martu S. Study on the TNF-α, IL-1 and IL-6 level in patient with chronic periodontitis and cardiovascular diseases. Rev. Chim (Bucharest) 2017; 68(3): 561-623.
http://www.revistadechimie.ro/pdf/43%20NICOLAICIUC%20O%203%2017.pdf

2.Ancuța C, Chirieac R, Ancuța E, Țănculescu O*, Solomon SM*, Fătu AM, Doloca A, Iordache C. Exploring the role of interleukin -6 receptor inhibitor tocilizumab in patients with active rheumatoid arthritis and periodontal disease. J Clin Med. 2021; 10(4): 878
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7924637/

II.4.1 Effects of combining classical periodontal therapy and antilipemic treatment in patients with chronic periodontitis and cardiovascular diseases

Aim of the study

This study aimed to assess levels in serum and crevicular fluid (GCF) of TNF-α, IL-1β and IL-6, to clarify the possible link between periodontitis and hyperlipidemia, as well as the effects of conventional periodontal treatment by scaling and root planing on these pro-inflammatory molecules.

Materials and method

The study included a total of 40 subjects divided into two main groups: the study group (n=26) and control group (n=14). The cases included patients with atherosclerosis with prescribed diet (D) or antilipemic therapy with a drug from the statin group (S). Controls (C) were selected from systemically healthy subjects with chronic periodontitis.

Exclusion criteria were represented by any other systemic diseases that affect lipid metabolism (affected glucose tolerance, diabetes mellitus, metabolic syndrome, or other endocrine diseases, nephritic syndrome, chronic renal disease and cardiovascular disease), any treatment antilipemic drug for more than 1 month, any current hormone replacement therapy, three-fold elevation of liver enzymes, which received no periodontal treatment in the last 6 months, and any systemic antibiotic administration during the last 3 months. Smokers and former smokers were also excluded.

Each patient completed a questionnaire that included data on oral hygiene, diet, medication history, current medication. All data obtained from clinical history and clinical and laboratory examinations were recorded in individual files.

Cases were submitted to a comprehensive periodontal examination which included radiographs and periodontal diagnosis. Oral health status of the control group was verified by clinical examination. Measurements of periodontal parameters, periodontal probing, BOP, plaque index, calculus index, tooth mobility and furcation lesions were standardized to the examiners.

The study was conducted according to the Helsinki Declaration. All participants gave their informed consent. All analyses were performed in blind.

Metabolic parameters were analysed at baseline and at 3 months after periodontal therapy. Blood samples were taken to measure levels of triglycerides (TRG), total cholesterol (TC), low density lipoproteins (LDL), high density lipoprotein (HDL) and very low density lipoprotein (VLDL). Samples were obtained after a fasting period of 12 hours from the ante-cubital vein. Serum lipid levels were determined by routine enzymatic methods.

Analysis of IL-1β, IL-6 and TNF-α in serum and in crevicular fluid was conducted at the beginning of the study and at 3 months after periodontal therapy.

For cytokine analysis 5ml of venous blood were drawn from an antecubital vein using a blood collection tube. Blood was allowed to clot for 30 minutes on ice and centrifuged for 10 minutes at 3000 rpm prior to placing the serum in 0.5 ml aliquots polypropylene tubes, which were stored at -40^0C until biochemical analysis. For laboratory assessments of serum the Enzyme-Linked Immunosorbent Assay (ELISA) was used. Results are expressed in pg/ ml. The lower limits of detection were <7, <2, and <9 pg/ml for IL-1β, IL-6 and TNF-α, respectively. Crevicular fluid samples were collected from sites of disease (PPD ≥4 mm). TNF-α, IL-1β and IL-6 concentrations were determined using ELISA tests.

Periodontal therapy included manoeuvres of scaling and root planing (Full-mouth disinfection), with ultrasonic and manual instruments, performed in the Clinic of Periodontology, Faculty of Dental Medicine of the "Grigore T. Popa" UMPh, Iaşi.

Post-treatment evaluation of local and systemic inflammatory status was carried out at 3 months after periodontal therapy. At this stage, specialized clinical assessments of patients, with periodontal indices re-determination were performed. The quantification of cytokines was performed in serum by ELISA.

Statistical analysis was performed using SPSS 20.0 (IBM, Armonk, NY, USA) and $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Twenty-six patients with atherosclerosis, aged 39-62 years (13 women and 13 men) and 14 systemically healthy control subjects aged between 30 and 51 years (7 women and 7 men) participated in the study. There were no significant differences related to age and gender ($P > 0.05$).

Eight patients in the test group received a dose of 10mg or 20 mg atorvastatin for 1 month. At the end of the first month, the lipid levels of statin patients were reassessed for possible need for dose adjustment. However, it has been found there is no need to change the dose of the statin. Thus, statin dose of the studied population was constant throughout the study. Also, all patients said they have complied with the doctor's recommendations during the study.

For groups of atherosclerosis, body mass index (BMI) ranged between 22.24 and 41.38 and for systemic group healthy BMI value ranged between 18.00 and 30.60 at baseline. Groups S and D had a greater value of BMI compared to the C group ($P < 0.05$ and $P < 0.01$, respectively) at baseline and at the end of the study period ($P<0.05$ and $P<0.01$, respectively).

Table II.31. Clinical and serum parameters on group studies*									
	C (n = 14)			D (n = 14)			S (n = 12)		
	Baseline	3M	P Value	Baseline	3M	P Value	Baseline	3M	P Value
PI	1.05 (0.38–3.20)	0.15 (0.13–1.90)	0.000	1.18 (0.31–2.90)	0.20 (0.11–2.01)	0.002	1.48 (0.28–2.84)	0.25 (0.11–1.20)	0.000
GI	1.10 (0.25–2.13)	0.12 (0.02–0.65)	0.000	0.91 (0.33–2.20)	0.12 (0.07–1.10)	0.000	0.90 (0.25–2.10)	0.16 (0.11–1.17)	0.001
PPD(mm)	2.64 (1.00–4.26)	2.10 (1.90–3.40)	0.015	3.06 (1.06–0.13)	1.31 (1.12–2.83)	0.049	1.92 (1.77–3.87)	1.30 (1.84–2.67)	0.000
BOP (%)	51.02 (8.38–100)	44.00 (0.23–100)	0.000	51.18 (3.34–100)	15.21 (6.25–44.00)	0.000	91.50 (0.60–100)	15.60 (5.79–32.69)	0.001
CAL(mm)	3.18 (1.16–7.13)	3.09 (2.06–5.60)	0.264	3.14 (1.25–4.38)	3.04 (1.94–4.05)	0.758	3.33 (1.21–4.89)	2.91 (1.93–4.95)	0.085
BMI (kg/m^2)	24.90 (18.00–30.60)	25.40 (19.20–32.20)	0.777	28.65 (22.24–36.87)	28.50 (17.75–38.70)	0.218	28.77 (24.19–41.38)	29.57 (23.28–38.42)	0.983
TC/HDL	3.52 (2.09–4.75)	3.88 (2.26–5.88)	0.114	5.26 (3.16–7.46)	5.12 (2.20–7.26)	0.244	4.66 (2.97–9.10)	4.71 (2.89–8.54)	0.108
TC(mg/dl)	163.00 (90.00–190.00)	162.00 (133.00–209.00)	0.324	221.50 (130.00–271.00)	213.50 (144.00–269.00)	0.856	218.00 (144.00–347.0)	204.50 (123.0–298)	0.594
LDL(mg/dl)	96.50 (31.80–124.40)	98.60 (27.70–148.20)	0.717	150.00 (59.00–192.80)	138.20 (62.00–193.00)	0.834	146.90 (78.60–216.00)	130.10 (64.00–202.40)	0.348
HDL(mg/dl)	46.00 (33.00–72.00)	47.00 (31.00–62.00)	0.102	41.00 (30.00–61.00)	41.00 (27.00–64.00)	0.834	44.00 (19.00–67.00)	45.00 (34.00–77.00)	0.463
VLDL(mg/dl)	17.40 (8.00–43.20)	23.20 (10.50–41.00)	0.019	25.00 (10.20–74.00)	30.80 (11.00–77.00)	0.209	23.30 (9.00–147.00)	38.20 (13.80–114.60)	0.413
TG(mg/dl)	84.00 (38.00–203.00)	98.00 (44.00–210.00)	0.102	124.00 (50.00–367.50)	153.50 (53.00–286.00)	0.549	117.00 (45.00–686.00)	137.50 (71.00–307.0)	0.656

***Values are expressed as median (min–max)**
C=control group; D=diet group; S=statin group; 3M= evaluation at 3 months after periodontal treatment; PI=plaque index; GI=gingival index; PPD=periodontal probing depth; BOP=bleeding on probing index; CAL=clinical attachment loss; BMI=body mass index; TC=total cholesterol; LDL=low density lipoproteins; HDL=high density lipoproteins; VLDL= very low density lipoproteins; TG=triglycerides.

GCF and serum levels of TNF-α, IL-1β and IL-6 of the study groups are shown in Table II.32. In all groups there was a significant decrease in TNF-α, IL-1β and IL-6 from baseline and the most significant decreases was recorded for IL-1β for statin group ($P < 0.01$).

Table II.31 displays the periodontal clinical parameters and serum lipids in study groups according to the study periods. For the C, S and D groups there were significant differences in parameters between the baseline and 3 months evaluation, except CAL (Table II.32).

Significant decreases were found in the crevicular fluid for all cytokines (Table II.32). The most obvious decrease ($P = 0.001$) was for IL-6 in the group of statins.

Table II.32. Serum and GCF cytokine values									
	C (n = 14)			D (n = 14)			S (n = 12)		
	Baseline	3M	P Value	Baseline	3M	P Value	Baseline	3M	P Value
Serum (pg/ml)									
TNF-α	15.92 (1.70–178.94)	10.61 (0.75–93.06)	0.043	16.07 (1.70–732.66)	10.16 (0.14–130.63)	0.039	18.39 (2.52–177.51)	9.73 (4.90–68.92)	0.029
IL-1β	2.95 (0.90–27.08)	1.85 (0.53–20.40)	0.020	6.52 (0.73–31.40)	3.02 (1.00–15.34)	0.015	4.35 (0.23–30.75)	1.62 (0.11–12.35)	0.007
IL-6	5.83 (3.41–62.43)	4.12 (3.52–36.15)	0.050	8.26 (3.41–117.18)	5.12 (3.46–7.38)	0.001	8.01 (0.49–107.24)	6.05 (3.21–10.71)	0.023
GCF (pg/ml)									
TNF-α	0.51 (0.42–0.62)	0.49 (0.34–0.50)	0.050	2.41 (2.00–2.42)	0.52 (0.31–1.22)	0.005	0.56 (0.1–5.23)	0.20 (0.1–1.12)	0.039
IL-1β	2.11 (0.54–63.49)	2.79 (1.53–22.35)	0.230	2.35 (0.98–100.27)	3.00 (0.63–33.44)	0.968	3.91 (1.16–62.2)	2.36 (1.32–48.54)	0.180
IL-6	0.82 (0.12–2.57)	0.61 (0.49–1.43)	0.039	1.06 (0.98–1.14)	0.20 (0.81–1.67)	0.021	1.47 (1.01–1.85)	0.14 (0.07–0.19)	0.001
***Values are expressed as median (min–max)** **C=control group; D=diet group; S=statin group; 3M= evaluation at 3 months after periodontal treatment; GCF=gingival crevicular fluid; TNF-α = tumor necrosis factor -alpha; IL-1β =interleukine-1 beta, IL-6= interleukine-6.**									

Discussion

Periodontitis is an infectious disease caused by predominantly anaerobic Gram-negative bacteria, and causes exacerbation of systemic and local proinflammatory molecules such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1 beta (IL-1β), RANKL and IL-6.

These cytokines generate an increased mobilization of fats from the liver and adipose tissue (Sun et al., 2016) and enhance the binding of low density lipoprotein (LDL) to the endothelium and smooth muscles.

There is evidence that infections may be responsible for the accelerated development of atherosclerosis (Pfeiler et al., 2019). The association between periodontal infection and an increased risk of cardiovascular disease was highlighted by several researchers (Greenblum et al., 2012).

Impaired lipid metabolism may play an important role in the association between periodontitis and cardiovascular diseases. Patients diagnosed with atherosclerosis presented significantly higher periodontal parameters values than subjects with of a normal metabolic status (Awartani & Atassi, 2010).

Furthermore, it has been shown that an improved periodontal health may affect the metabolic control of hyperlipidemia and can be considered as an adjunct to the standard of care of the patient with atherosclerosis (Fentoglu et al., 2011).

At baseline, lifestyle changes such as diet modification and exercise - which are important components of treatment of lipid disorders - have been recommended in addition to antilipemic drug therapy. All patients said they have complied with the doctor's recommendations during the study, but further monitoring was not performed. Because it was not possible to measure fat mass during routine medical consultation, it was decided to use BMI as a substitute for changes in body composition. According to current results,

periodontal treatment generally resulted in a slight decrease in atherogenic lipid profile with diet group (D).

In the statin group (S), there were also decreases in serum total cholesterol and LDL. In agreement with these findings, a study (Yudkin et al., 2000), which was conducted in hyperlipidemic patients, suggested that a group of antilipemic drugs resulted in significant decreases in serum total cholesterol and LDL, 3 months after completion of periodontal treatment.

Bleeding on probing (BOP) was significantly higher in S group than C and D. This result can be interpreted by the fact that the extent of damage to lipid metabolism may be associated with periodontitis when taking into account both inflammatory components.

According to current findings, the S group had serum and GCF IL-6 levels higher than D and control groups. In addition, increased levels of BOP were observed in S group compared to groups C and D.

According to the results, there have been significant decreases in IL-6 serum levels at the 3 months assessment of atherosclerosis groups. When taking into account that IL-6 could play a key role in the development of coronary artery disease (through a number of different mechanisms: metabolic, endothelial and coagulation) and there is a close relationship between circulating concentrations of CRP, IL -6 and TNF-α and serum lipid components (Yudkin et al., 2000), the role of periodontal treatment in patients with atherosclerosis becomes important.

Several cytokines play a role in the pathogenesis of both coronary heart disease and periodontitis. These include interleukin-1, interleukin-6, interleukin-8, tumor necrosis factor, intercellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin. Interleukin-6 is involved in promoting coagulation, which may lead to the development of atherosclerosis. In a prospective study of 14 916 apparently healthy subjects, levels of IL-6 in 202 subjects who had a subsequent heart attack were higher than those of 202 control subjects, during a follow-up of 6 years (1, 8 to 1.5 pg/ml, $P = 0.002$) (Ridker et al., 2000). This indicates that the levels of interleukin-6 may be a predictor of future risk of heart attack in apparently healthy subjects.

Severe forms of periodontitis can lead to a state of systemic inflammation characterized by high serum levels of IL-6. One study showed that both subjects with coronary artery disease and periodontitis had significantly higher serum levels of IL-6 in comparison to subjects with coronary artery disease who did not have periodontitis ($P < 0.05$) (Higashi et al., 2009). The results of studies on the effect of periodontal intervention therapy on serum interleukin-6 are not consistent. Most studies have taken confounding factors such as gender, age, smoking habits and medical history into account.

The immune system plays an important role in the pathology of periodontal disease, because most of the genes responsible for the development of periodontitis are related to the immune response. These include genes that affect IL-1, IL-6, TNF-α, IL-10, Fc-gamma receptor, CD14.

The effects of IL-6 and TNF-a on lipid metabolism could still affect endothelial nitric oxide generation as a result of elevated non-esterified fatty acids. The effect of IL-6 on platelets, fibrinogen and coagulation and of TNF-α in the expression of endothelial cells, adipose tissue and hepatocytes plasminogen activator inhibitor, can lead to a pro-coagulant state in such subjects. Therefore, the reduction of inflammatory mediators such as TNF-α and IL-6 (serum and/or GCF), which are also associated with atherosclerosis and periodontitis, may provide an additional contribution to the bidirectional relation between periodontitis and atherosclerosis.

Conclusions

Even some drug strategies can prove to generate unfavourable effects on periodontal tissue (such as anticonvulsants or cyclosporine A), others can offer synergic activities in the periodontal treatment. Combining periodontal therapy and antilipemic treatment can provide beneficial effects on metabolism and control of inflammatory atherosclerosis by lowering serum proinflammatory cytokines. The same benefit was also observed in patients with THS and osteoporosis or DMARDS and rheumatoid arthritis.

II.4.2 Exploring the role of Interleukin-6 receptor inhibitor Tocilizumab in patients with active rheumatoid arthritis and periodontal disease

Aim of the study

We conducted a study with the aim to assess the influence on the periodontal status of weekly subcutaneous administration of tocilizumab in a local group of patients with rheumatoid arthritis and chronic periodontitis.

Materials and method

We performed a prospective longitudinal study in fifty-one patients with moderate to severe RA and insufficient response to either conventional synthetic or biologic disease-modifying anti-rheumatic drugs (DMARDs), starting TCZ according to the local recommendation for biologic and targeted synthetic therapy aligned with European League Against Rheumatism (EULAR) consensus statement and guidelines. We performed extensive rheumatologic and full mouth assessments at baseline (before the first administration of TCZ) as well as after 3 and 6 months of therapy.

The study methodology respected the Helsinki Declaration norms. The patients were aged 18 and older, able to give informed consent themselves and to participate in the study, and willing to forgo any optional examinations. Several exclusion criteria were applied before enrolment in this study because of their potential interference with a correct evaluation of periodontal status, as follows: ex or current smokers, pregnant and breastfeeding women, patients with diabetes mellitus, implants, poorly fitting fixed and/or removable prosthodontics and fewer than eight evaluable teeth, patients receiving systemic or local antimicrobials, antiplatelet drugs, any type of anti-inflammatory medication or periodontal therapy within the previous 3 months. A total of sixty-eight patients were eligible for and received TCZ for their active RA; however, among them, seventeen had no oral issues at baseline evaluation and were excluded from the study.

RA-related variables comprised clinical (tender and swollen joint count based on a 28-joint assessment, 0–10 cm visual analogue scale, VAS, pain), inflammatory tests (erythrocyte sedimentation rate, ESR, and C-reactive protein, CRP) as well as disease activity scores calculated on DAS28-CRP (Disease Activity Score on 28 joints using C-reactive protein) and SDAI (Simplified Disease Activity Index) were performed at all three visits. DAS28-CRP was calculated with a formula that considered the tender and swollen joints, the patient's general assessment of their condition scored on a visual analogue scale (VAS), and CRP. DAS28-CRP comprises four categories: remission (DAS28-CRP < 2.3), low ($2.3 \leq$ DAS28-CRP < 2.7), moderate ($2.7 \leq$ DAS28-CRP < 4.1), and high disease activity ($4.1 \leq$ DAS28-CRP).

Designed as the numerical sum of five outcome parameters (tender and swollen joints, patient, and physician global assessment of disease activity on a 0–10 VAS and CRP level), SDAI score interpretation comprises also four categories: remission (0–3.3), low activity (3.4–11), moderate activity (11.1–26), and high RA activity (26.1–86). Serological biomarkers (rheumatoid factor, RF, and ACPA) were evaluated only at baseline.

The periodontal status was recorded on a periodontal chart displaying the following

clinical parameters for the entire dentition: number of present teeth, visible plaque index (VPI), gingival index (GI), bleeding on probing (BOP), probing pocket depth (PPD), and clinical attachment loss (CAL). The mean values were assessed using paired t -test for VPI, GI, BOP, PPD, and CAL.

Patients were instructed to maintain their oral hygiene habits throughout the 6 months of follow-up; furthermore, as we intended to assess the accurate effect of TCZ on periodontal status, any periodontal treatment was avoided.

Results

Demographics, rheumatologic and periodontal characteristics, as well as RA-related drugs (concomitant glucocorticoids and immunosuppressives) taken at baseline are summarized in Table II.33.

Table II.33. Demographic, rheumatologic, and periodontal characteristics at baseline

Parameters	Baseline values
Age (years; mean ± SD)	56.3 ± 15.7
Female (n, %)	46 (90.1)
Duration of RA (months; mean ± SD)	81.3 ± 68.9
DAS28-CRP (mean ± SD)	5.36 ± 1.67
SDAI (mean ± SD)	34.2 ± 16.3
Corticosteroids (n, %)	20 (39.21)
DMARDs (n, %)	46 (90.1)
ACPA levels (U/mL; mean ± SD)	239.7 ± 124.3
ACPA positivity (n, %)	32 (62.74)
RF levels (IU/mL; mean ± SD)	192.7 ± 85.3
RF positivity (n, %)	47 (92.15)
Serum CRP levels (mg/dL; mean ± SD)	15.3 ± 6.9
Number of present teeth (mean ± SD)	23.7 ± 3.4
GI (mean ± SD)	0.98 ± 0.12
% sites with plaque (mean ± SD)	32.4 ± 16.9
% sites with BOP (mean ± SD)	10.2 ± 8.6
PPD (mm; mean ± SD)	2.8 ± 0.4
% sites with PPD > 4 mm	12.7 ± 2.5
CAL (mm)	3.5 ± 1.2
% CAL >3 mm	12.5 ± 0.2

RA, rheumatoid arthritis; DAS28-CRP, Disease Activity Score on 28 joints using C-reactive protein; SDAI, Simplified Disease Activity Index; DMARDs, disease-modifying antirheumatic drugs; ACPA, anti-citrullinated protein antibodies; RF, rheumatoid factor; PD, periodontal disease; GI, gingival index; BOP, bleeding on probing; PPD, probing pocket depth; CAL, clinical attachment loss; n, number; SD, standard deviation; %, percent.

Most patients included in our study had seropositive established RA, with moderate to severe activity despite background medication. Eight patients (15.68%) received TCZ as their first biologic agent (bio-naïve), while the majority were bio-experienced patients, with failure (either insufficient response or adverse reactions) to previous biologics—15 (29.41%) to one biologic, 20 (39.21%) to two biologics, and 8 (15.68%) to three biologic agents.

We detected impaired oral health in all patients included in the final analysis, as follows: all had gingivitis (abnormal GI and increased prevalence of sites with BOP), and different degrees of chronic periodontitis (mainly level 1 and 2); advanced loss of attachment was reported in up to 23.52% of cases, while increased prevalence of sites with dental plaques in 21.56% of cases.

A closer look revealed a consistent positive correlation between the severity of chronic periodontitis, RA activity, and serum r2 = 0.71 ACPA concentrations (r1 = 0.81, p1 = 0.001, p2 = 0.002, respectively): the higher the RA activity and ACPA levels, the higher the PPD severity, with advanced CAL and tooth loss.

Changes in RA activity and periodontal status were reassessed after 3 and 6 months of TCZ; at follow-up visits, we reported significant improvement as compared to baseline ($p < 0.05$), although the results at 6 months were only slightly different from data obtained at 3 months ($p > 0.05$).

Patients displayed consistent improvements in clinical activity meaning a significant decrease in the number of tender and swollen joints, VAS pain, and morning stiffness as rapid as 3 months; as expected, clinical response was maintained 3 months later in all patients, at the final monitoring visit. Similarly, we reported a dramatic decline in inflammatory biomarkers (both ESR and CRP), as well as a considerable immunologic response, particularly for serum levels of ACPA, but also for RF (Table II.34).

Table II.34. Changes in rheumatoid arthritis (RA)-related parameters at 3 and 6 months after tocilizumab.

Parameter	Baseline	3 Months (V1)	6 Months (V2)	p -Value
DAS28-CRP (mean ± SD)	5.36 ± 1.67	3.39 ± 0.57	2.41 ± 0.19	*<0.05; **<0.05
SDAI (mean ± SD)	34.2 ± 16.3	18.1 ± 8.2	11.1 ± 4.3	*<0.05; **<0.05
Number of tender joints (mean ± SD)	12.31 ± 4.29	4.56 ± 1.31	3.55 ± 1.13	*<0.05; **NS
Number of swollen joints (mean ± SD)	10.01 ± 3.37	2.85 ± 4.22	1.50 ± 2.09	*<0.05; **NS
Pain VAS mm (mean ± SD)	82.7 ± 21.5	28.8 ± 23.2	16.3 ± 11.8	*<0.05; **<0.05
Serum anti-CCP titer (U/mL) (mean ±SD)	239.7 ± 124.3	192.6 ± 112.4	123.6 ± 101.6	*<0.05; **NS
Serum RF levels (IU/mL) (mean ± SD)	192.7 ± 85.3	164.8 ± 92.5 4.12 ± 0.92	151.7 ± 89.3	*NS; **NS
Serum CRP levels (mg/dL) (mean ± SD)	15.3 ± 6.9		3.92 ± 0.34	*<0.05; **NS
SD; standard deviation; DAS28-CRP, Disease activity score on 28 joints based on C-reactive protein; SDAI, Simplified Disease Activity Index; VAS, 0–10 cm visual analogue scale; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; V, visits; * V1 compared to baseline; ** V2 compared to V1; NS, non-significant (0.05).				

DAS28-CRP and SDAI strongly improved during monitoring visits reaching either low disease activity or, even, remission (EULAR responders) vs. baseline, irrespective of the severity of periodontitis.

Clinical data showed improvement in periodontal inflammation after only 3 months of TCZ and maintained over 6 months, as supported by an important decrease in gingival index and sites with bleeding of probing ($p < 0.05$). However, the improvement of specific periodontal parameters such as probing pocket depth becomes evident after prolonged treatment (6 months); overall, clinical attachment loss presented only slight changes without any statistical significance; teeth count and bacterial plaque scores were also not significantly influenced by medication ($p > 0.05$) (Table II.35).

Table II.35. Changes in PD-related parameters at 3 and 6 months after tocilizumab

Parameter	Baseline	3 Months (V1)	6 Months (V2)	p -Value
GI	0.98 ± 0.12	0.85 ± 0.17	0.81 ± 0.18	*<0.05; **NS
% sites with plaque	32.4 ± 16.9	30.5 ± 14.2	30.2 ± 15.8	*NS; **NS
% sites with BOP	10.2 ± 8.6	7.3 ± 6.1	6.5 ± 6.8	*<0.05; **NS
PPD (mm)	2.8 ± 0.4	2.1 ± 0.12	2.1 ± 0.09	*<0.05; **NS
% sites with PPD ≥ 4 mm	12.7 ± 2.5	7.8 ± 3.9	6.1 ± 3.6	*<0.05; **NS
CAL (mm)	3.5 ± 1.2	2.58 ± 0.30	2.55 ± 0.31	*<0.05; **NS
% sites with CAL ≥ 4 mm	12.5 ± 0.2	11.2 ± 0.4	11.3 ± 0.9	*<0.05; **NS
GI, gingival index; BOP, bleeding on probing; PPD, probing pocket depth; CAL, clinical attachment loss; * V1 compared to baseline; ** V2 compared to V1; p > 0.05 non-significant (NS); V1 and V2, visit 1 and 2, respectively.				

No significant correlations between changes in periodontal parameters and changes in RA activity were described in our study ($p > 0.05$). We assumed that all the modifications in the degree of local gingival and periodontal inflammation is related to IL-6 blockade as no local periodontal treatment was allowed during follow-up.

Discussion

We aimed to assess the influence of the IL-6 receptor inhibitor on periodontal status in active RA associated with periodontitis, assuming that TCZ might be able not only to improve clinical and biochemical RA-related parameters but also to ameliorate chronic periodontitis as a result of decreased IL-6 in the periodontal microenvironment via declining systemic inflammation.

Although our target is to demonstrate the ability of TCZ to modulate periodontal inflammation and subsequent damage, firstly, we emphasized its role in controlling RA activity. We reasonably confirmed a consistent response to TCZ in real-life settings, which was achieved in as rapid as three months and continued after six months of therapy, irrespective of background medication and clinical scenario (mono- or combined therapy, bio-naïve or bio-experienced patients); it is more than clear that even in the short-term, IL-6 blockade displays significant clinical, biological, as well as serologic disease improvement.

Although we found no consistent difference in clinical response in seropositive vs. seronegative RA, we noticed a significant impact on ACPA serum concentration after six months, which is an improvement that parallels the decrease in periodontal inflammation, suggesting a role of IL-6 in both systemic and local inflammation (synovial and periodontal) and the potential implications via citrullination. Therefore, our results stand by as a proof of the effectiveness of subcutaneous TCZ in managing inflammatory and immune pathways in RA.

We also focused on the magnitude of compromised oral health in RA; most patients in our initial group presented a high rate of mild and severe periodontal disease, validating/reinforcing the already known risk of periodontitis in such patients, particularly in established, longstanding disease (Bartold & Lopez-Oliva, 2020). We have included in the final analysis only those cases with overt periodontal disease, meaning that up to 75% had at baseline altered periodontal status in a group of consecutive patients starting TCZ for their active disease. Indeed, reviews and meta-analyses have already discussed periodontal disease in various RA settings (independent of age, disease duration, serology profile, and disease activity) compared to general population (de Molon et al., 2019; Gómez-Bañuelos et al., 2019).

We identified excessive gingival involvement confirmed by an increased percentage of sites with plaques and inflammation and abnormal periodontal status (e.g., increased probing depth, clinical attachment loss) supporting data from the literature (Qiao et al., 2020). Moreover, we recognized positive correlations between the severity of periodontitis, inflammatory parameters (especially CRP), serology (ACP status and titres), and RA activity; indeed, recent data suggest a worse periodontal status in active untreated RA, and higher CRP if RA is associated with severe periodontitis (Mankia et al., 2019). Finally, it seems that ACPA-positive patients had severely impaired periodontal health, while disease activity correlated with periodontitis degree as well (Äyräväinen et al., 2017).

Finally, we demonstrated that short-term tocilizumab significantly reduced gingival as well as periodontal inflammation as supported by decreased levels of gingival index, bleeding on probing, and probing pocket depth, paralleling the articular improvement. Indeed, only minor changes in clinical attachment loss were detected in our enrolled patients, and the supragingival plaque remained stable after 3 and 6 months of biological treatment ($p > 0.05$).

A closer look at recent data definitely emphasizes the dual effects of early and

aggressive RA treatment with biologic and non-biologic drugs (Janus kinase inhibitors, JAK inhibitors) on articular as well as comorbid periodontal disease (Thilagar et al., 2018). It is widely accepted that TNF and IL-6 receptor inhibitors are able to ameliorate oral health in active RA, as reflected by clinical, biological, and even serological RA biomarkers (Jung et al., 2018).

Although there are controversial effectiveness signals with TNF inhibitors in improving chronic periodontitis (Choi et al., 2016), all papers about anti-IL-6 therapy clearly demonstrated articular, systemic, and also periodontal benefits with TCZ without any periodontal specific treatment (Kobayashi et al., 2010; 2018).

An interesting trial compared periodontal condition in patients with RA and periodontitis before and after biological therapy in two cohorts: one under tocilizumab and the other receiving medication with TNF inhibitors (Kobayashi et al., 2015). After 6 months, both tocilizumab and TNF inhibitors demonstrated a consistent improvement of oral health with significantly reduced periodontal inflammation (gingival index, bleeding on probing, and probing depth) compared to baseline, with similar results in both cohorts unless there was a greater decrease in gingival index and less gingival inflammation with tocilizumab; however, plaque levels remained the same irrespective of medication, while periodontal clinical attachment loss decreased only after TCZ but not after TNF inhibitors (Kobayashi et al., 2014; 2015). These observations were partially supported by the results of another study about an excessive inflammatory response against oral pathogens essentially based on high levels of IL-6 (Nibali et al., 2011).

Recent meta-analyses reviewed the most important studies on TNF and non-TNF biologics in patients with RA and PD (Rinaudo-Gaujous et al., 2020; Qiao et al., 2020). The critical difference between the class of TNF inhibitors and TCZ or B-cell depletive agent rituximab is that infliximab, an anti-TNF monoclonal antibody, may negatively address gingival inflammation although it may also improve alveolar bone destruction (Kobayashi et al., 2014), resulting in a dissociated response for patients with severe periodontitis, while both tocilizumab and rituximab associate with significant a down regulation of gingival inflammation and damage in RA associated with periodontitis (Coat et al., 2015)

Additional research is necessary to clearly differentiate between the direct effects of TCZ on local periodontal inflammation and IL-6 or its receptor levels in the gingival crevicular fluids and periodontium of patients and the indirect effect via dramatically decreasing systemic inflammation, which may impact also oral health (Kobayashi et al., 2014). Indeed, numerous studies indicated a rapid and significant decline in typical inflammatory parameters (ESR and CRP), but also in serological RA biomarkers (RF, ACPA) as well as inflammatory cytokines (TNF, IL-6) and mediators (serum-amyloid A, matrixmetaloproteinases 1, 3), supporting the indirect role of TCZ in periodontitis (Kobayashi et al., 2014; 2015).

Conclusions

In our study, we assessed specific gingival and periodontal parameters before and after short-term TCZ therapy. We demonstrated successful RA as well as periodontal outcomes with TCZ and independent of potential confounding factors (such as smoking, diabetes, haematological conditions, sex steroid hormones elevations, pharmacological agents) related to periodontal disease, as such patients were excluded from the final analysis.

We concluded that tocilizumab decreased gingival inflammation since no periodontal therapy was permitted and the dental hygiene behaviour remained unchanged in our enrolled patients.

Unfortunately, we were not able to assess either the serum or gingival crevicular fluid levels of IL-6 or its receptor in all patients; we assumed that tocilizumab indirectly

contributes to modulate local (gingival and periodontal) inflammation by limiting systemic inflammation. Indeed, the biofilm plaque accumulation was not consistently diminished with tocilizumab, and we were not able to depict a spectacular impact on clinical attachment loss, but we arrived to demonstrate a positive effect of IL-6 blockade on exuberant gingival inflammation.

Further studies are necessary to confirm the benefits of Il-6 inhibitors in larger populations and longer follow-ups also focusing on IL-6/IL-6 receptor levels in gingival crevicular fluid.

SECTION II

FUTURE EVOLUTION AND DEVELOPMENT PLANS UPON CAREER AND RESEARCH ACTIVITY

Throughout my 30-year career as a dentist and university teacher, I have tried to harmoniously combine clinical practice with the teaching act and to bring in this equation scientific research as a source of information to substantiate both the medical act and teaching.

I considered that the professional evolution is based on a constant effort to update the acquired knowledge, through individual learning and by participating in national and international scientific events. Once I got in touch with the newcomer in the field, I tried to acquire technical skills so as to implement it both in day-to-day practice and in the act of theoretical and practical teaching.

In my future activity I will continue to gradually improve my skills in the professional, academic and scientific domains and carefully balance all areas of my work. As medical knowledge continuously increases, I will continue to learn based on individual study and by attending professional national and international conferences and scientific meetings organized by prestigious dental associations such are European Federation of Periodontology - EFP, Romanian Society for Dental Education - ADRE, Romanian Society for Oral Rehabilitation - SRRO, National Union of Dental Associations – UNAS in which I am also a member.

Regarding novelties, I will make efforts to stay informed with the latest discoveries in periodontology in order to update both the diagnostic protocols I use as well treatment protocols, according to global trends. I will also focus my training on those fields that I consider soft spots, trying to enlarge my horizons.

Beside adaptation, other outcomes of evolution are cooperation and coevolution. It is mandatory to cooperate with colleagues and sustain them, periodontologists or from other dental and medical specialties for both beneficial and development.

At national and international level, as member of the Romanian Society of Periodontology I will seek knowledge dissemination and collaboration with peers worldwide.

Some of the research subjects that I am interested in developing and represent ongoing and future studies will be guided toward:

- Carry on and settling the research of the periodontally challenged patients on the level of oral cavity status and relationships with systemic diseases supporting the scientific research reports;
- Dissemination of results in ISI / indexed international databases journals;
- Continuing and expanding the collaboration with the preclinical disciplines such as Immunology, Biochemistry, Medical Genetics, Pharmacology and other dental disciplines (prevention, cariology, endodontics, pedodontics, orthodontics, prosthetics), and also with other medical specialities (cardiology, nephrology, diabetology, oncology, gastroenterology, etc) addressing new proposals that could provide real funding through joint research projects;
- Attracting funds to improve the means of research at the Periodontology discipline;
- Expanding collaboration with other research laboratories outside the faculty;
- Organizing and participating in national and international scientific events;
- Expanding periodontology research through participation in national grants;
- Developing applications for international research programs and grants;

- Organizing student scientific events in order to involve and include them in the research activity and activities of disseminating results;
- Involvement of residents in research teams on the proposed topics.

II.1. FUTURE DIRECTIONS IN RESEARCH ACTIVITY

The human body is very complex and continuously challenging, therefore only interdisciplinary research can integrate data, methodologies, perspectives, and concepts from various fields to characterize its homeostasis.

The major path of my future academic research will concern directions and areas that are already in progress, involving activities that made the fundamental of my last five years preoccupations, such are the diagnosis and complex treatment of periodontal diseases but also to explore new research directions such are:

- **Evaluation of the link between various infectious diseases such as Hepatitis C Infection or Human Papilloma Virus Infection and Periodontal Disease**

Hepatitis C virus (HCV) infections could have an important impact on the oral health status of patients, favouring conditions such as periodontal disease and oral cancer. The changes caused by the infection in the subjects' immune system, diet, and lifestyle can facilitate the development of oral conditions such as periodontal disease. Important changes also occur in the composition of the infected patients' saliva and gingival fluid. HCV-infected patients need to be carefully monitored in terms of oral health since the infection with the virus can result in oral complications. I also intend to pursue upon the research on the presented directions as well as to extend the area of interest to other innovative diagnostic systems in the context of the fulminant advances in the medicine field.

Human Papilloma Virus (HPV) localizes to inflammatory periodontal tissue and is thought to infect basal keratinocytes in the ulcerated gingival sulcus epithelium. Inflammatory periodontal pockets might function as a reservoir for HPV. Even if the physiopathologic interactions between HPV and periodontal pathogens remain unclear, oral HPV infection may be associated with a specific oral microbiota. HPV and periodontal disease may contribute to the onset of oral cavity cancers. The literature data suggests that oral HPV may be associated with periodontal disease. In order to clarify the relationship between oral HPV and periodontitis, the effects of various clinical risk factors for HPV DNA prevalence should be investigated. Moreover, methods of sampling that can directly detect HPV DNA in inflammatory periodontal tissues should be further investigated. Studying this relationship is important since periodontitis might help to identify the risk for oral HPV infection and also potential HPV-related oropharyngeal cancers.

- **Expanding research on the role played by periodontal inflammation in the pathologic mechanism of different systemic diseases**

As Tellier said, "*The gingival-dental region is a small scene in which many great dramas of pathology are played in a narrower setting, the consequences of which are felt in the general life of the organism and have more important repercussions than is usually thought on human teeth".*

Disturbance on human microbiota colonizing the various body sites has been implicated in a wide range of microbiome-related inflammatory diseases. Among those, periodontal diseases are complex polymicrobial inflammatory diseases associated with dysbiosis of the dental biofilm that induces a long-lasting chronic inflammation of the periodontal supporting tissues, leading to alveolar bone destruction, and eventual tooth loss.

Over the years, strong evidence has accumulated to indicate that the pathogenic microbiota and the chronic inflammation established in periodontitis contribute to the onset and/or progression of several systemic inflammatory diseases such as cardiovascular diseases, diabetes, obesity, metabolic syndrome, respiratory disease, cancer, chronic kidney disease (CKD) and rheumatoid arthritis (RA). Most research on the periodontitis-systemic disease relationship, however, has not determined causality, and the link between these diseases are bi-directional associations.

There are several biologically plausible mechanisms to support these associations. I aim to develop research on the direct and indirect effects of circulating oral bacteria recognized as being able to enter the circulation and cause bacteraemia by actively crossing the periodontal epithelium, or by being inoculated through mechanical procedures, including periodontal debridement, flossing and brushing. Periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans, Treponema denticola* and *Porphyromonas gingivalis* are also capable of invading endothelial cells, and they have been detected in atherosclerotic plaques, heart valves, aortic aneurysms, carotid and coronary vessels.

I also aim to continue investigating the effects of inflammatory mediators and/or immune complexes from infected/inflamed periodontal tissues on other body sites as this is one of the main mechanisms that contribute to systemic inflammation.

Studies in a variety of animal models have demonstrated that recurrent bacteraemia or oral administration with *P. gingivalis* can enhance atherogenesis. Of interest, *P. gingivalis* is so far the only bacterium capable of causing enzymatic citrullination of peptides with subsequent development of anti-citrullinated peptide auto-antibodies, a major etiopathologic event in RA. Bacterial by-products, particularly LPS from the predominant Gram-negative periodontal biofilm may also contribute to systemic inflammation. Studies have showed a significant increase in the levels of systemic endotoxin and in hyperactivity of circulating neutrophils following 21 days of dental plaque accumulation (experimental gingivitis). After treatment of gingivitis, a reduction of endotoxemia to baseline levels was observed.

Alternatively, data have suggested that the inflammatory response to periodontal bacteria at the inflamed periodontal tissues represents a source of persistent chronic systemic inflammation. Pro-inflammatory mediators and biomarkers are significantly more elevated in serum and gingival crevicular fluid of individuals with periodontitis compared to periodontally healthy individuals. In addition, periodontal treatment generally lowers most of these mediators.

- **Investigation of the link between intestinal microbiome and periodontal disease and the potential therapeutic role of probiotics**

Disturbance on human microbiota colonizing the various body sites has been implicated in a wide range of microbiome-related inflammatory diseases. Among those, periodontal diseases are complex polymicrobial inflammatory diseases associated with dysbiosis of the dental biofilm that induces a long-lasting chronic inflammation of the periodontal supporting tissues, leading to alveolar bone destruction, and eventual tooth loss.

Intestinal Microbiome

One possible mechanism linking periodontitis and inflammatory systemic diseases would be through a disturbance of the gut microbiome by a long-term, orally ingested high dose of periodontopathic microorganisms. Based on this hypothesis, individuals with chronic periodontal diseases would eventually establish a disturbed gut microbiome commonly seen in individuals affected by systemic inflammatory diseases. In fact, the novel pathogenesis model of periodontitis (the 'keystone-pathogen hypothesis') proposes that periodontal pathogens can orchestrate inflammatory periodontal disease by remodelling a symbiotic

periodontal microbiota into a dysbiotic one, as demonstrated in animal model studies. However, no clinical studies in humans have evaluated the ability of periodontal pathogens to cause a dysbiosis in the gut microbiome. So far, only two studies in mice have shown that oral administration of *P. gingivalis* induces increased local and systemic inflammation, and significant changes in the gut microbiota composition. Furthermore, the gut microbial profile of systemically healthy individuals with periodontal diseases has not

There are studies that show individuals with periodontal diseases present a less diverse gut microbiome consistent with other systemic inflammatory diseases.

Understanding the key factors that allow gut colonization of oral pathogens might offer the possibility in stratifying patient groups in which presence of periodontitis is a significant risk factor for the development of gastrointestinal inflammatory disease; nevertheless, the mechanisms of gut microbiota dis-homeostasis involved in periodontitis evolution require further investigations. Beyond risk assessment and personalized prevention, one can conceivably speculate that new directions open up the possibility to investigate novel biomarkers and therapeutic interventions in colitis. Whether gut colonization with specific oral microorganisms can serve as a disease biomarker for colitis, remains to be explored. Similarly, targeting oral microorganisms in the treatment and/or prevention of gut inflammation may also be an important therapeutic avenue to consider. While the clinical and therapeutic potential of this work can only be speculative at this point, future findings could demonstrate novel concepts and mechanisms by which oral inflammation and oral microbes are connected to gastrointestinal disease.

Probiotics

The results of the animal and clinical periodontitis studies support the potential usage of probiotics in the adjunctive treatment of periodontitis and that probiotics may offer a low-risk, inexpensive, easy to use prevention or treatment option for the management of periodontal disease. Further research needs to focus on specific probiotic strains, doses, delivery methods, treatment schedule, mechanisms of action, safety and how to maintain the results of the probiotic interventions.

- **Periodontitis and respiratory disease**

The evolution of the pulmonary diseases can be affected by both infectious and inflammatory diseases, such as periodontitis. The periodontal pathogens present in the periodontal pocket can be aspirated into the lower airway. Epithelial sensitization and the hematogenous spread of the proinflammatory mediators such as cytokines and MMP produced in the inflammatory periodontal tissue can increase the global inflammatory burden, exacerbating disease activity with diminished airflow. Still, there is a need for further research in order to clarify the pathobiologic ways behind this particular relationship. In addition, both periodontitis and the respiratory diseases represent important public health issues and there is need for additional measures to implement oral health prevention and health promotion measures for these respiratory diseases.

- **Immunological senescence – aging patient – periodontitis**

Immune Senescence

The elderly display higher susceptibility to infectious and inflammatory diseases, including periodontal disease. Aging is thought to affect the immun-inflammatory status and/or the regenerative potential of the periodontal tissue in a manner that increases susceptibility to periodontitis. This “age-altered susceptibility” hypothesis is consistent with findings of aging-related changes in immune and stem cell function that can potentially dysregulate immune responses and impair periodontal tissue repair. A fundamental cause for

immune senescence consists in the aging of hematopoietic stem cells, which affects both specific and non-specific immunity. The use of endogenous molecules that can both inhibit inflammation and promote its resolution appears to be both safe and effective and represents a direction which needs further investigation. For instance, because expression of developmental endothelial locus-1 is diminished under inflammatory conditions or in old age, restoring its levels by exogenous administration could therapeutically reinstate tissue homeostasis.

- **Aggressive forms of periodontitis – when to abandon teeth preservation and swich to implant treatment**

Evidence-based dentistry requires application of current evidence in making decisions about the care of individual patients. Long-term preservation of the periodontium is the main objective of periodontal therapy. Before a treatment plan is established, the diagnosis and etiologic factors of the disease as well as the prognosis of the remaining teeth should be determined while predicting the final functional and esthetic result. Tooth prognosis can be classified as good, fair, poor, questionable, hopeless and indicated for extraction.

In decision making related to tooth extraction are involved patient-related factors and tooth-related factors that I aim to investigate in order to configure a viable protocol to decide tooth extraction.

Patient-related factors and tooth-related factors are involved in decision making related to tooth extraction, factors that I aim to investigate in order to configure a viable protocol to decide tooth extraction.

- **Studies on periimplant tissue maintenance in perio-patients/ early molecular diagnosis of failure.**

Periodontal maintenance is a critical factor in the long-term success of both periodontal and dental implant therapy. Studies have shown both modern periodontal and dental implant therapies are effective in maintaining natural teeth and replacing lost teeth, respectively. However, without a regular program of clinical reevaluation, plaque control, oral hygiene instruction, and reassessment of biomechanical factors, the benefits of treatment often are lost and inflammatory disease in the form of recurrent periodontitis or periimplantitis may result. I aim to develop studies in order to establish the goals, types, and appropriate frequency of periodontal maintenance in periodontal and dental implant therapy, as well as the incidence and etiology of periimplant disease and strategies for management when recurrent disease develops during the maintenance phase of treatment.

- **Studies on failure/ success of surgical periodontal procedures**

Tissue regeneration represents an important surgical alternative in treating both soft and hard periodontal tissue defects. Nevertheless, this particular strategy encounters limitations in the current practice. As part of my future research interests, I intend to investigate novel materials and techniques for guided tissue regeneration (GTR), such as 3D and 4D methods that could widen the range of GTR indications and also provide an individual patient-tailored altenrnative of treatment. This particular approach could offer high benefits in tooth an implant maintenance and also quality of life.

II.2. FUTURE DIRECTIONS IN TEACHING ACTIVITY

Students

Improvement in teaching methods plays an important role in teaching activity of any university professor. Our main role is to teach the students but is of paramount importance

how we can do this. At proper age, they are avid of information and full of enthusiasm to achieve all new competences and skilling possible. It is up to us to direct this focus not only to periodontology. At the same time, it is possible to select since from this stage of evolution future researcher or at least the best future residents capable to have an excellent clinical evolution.

An important argument to an easiness of relationship with students are the over 60 bachelor thesis conducted under my supervision. Only one year after my debut in University career I supervised four such thesis.

Teaching the residents in Periodontology represents an important part of my daily activity as they are the next generations of periodontologists. Improving my professionalism, providing them detailed explanations, encouraging them to individual study, to attend as much as possible scientific meetings, lectures and courses, stimulating them to publish scientific papers and including them in research projects are some of the directions I follow.

Linking research to educational and medical activities requires either implementing research results in medical practice and educational process, coordination of research topics within student scientific meetings, the progress in the teaching stages according to the acquired skills and the existing opportunities, while increasing the scientific reputation of the department / faculty / university, as well.

A good training in periodontology entails the acquiring of rational diagnosis and cure skills, together with the ability to transmit the information, and that is why my future teaching directions include:

- Systematization of a teaching protocol for courses and clinical practice,
- The individualization of the didactic protocol - adaptation of teaching level, professional-scientific language used and terminology, according to the level of medical culture (both in dentistry and general medicine), year of study;
- The adaptation of the didactic material - the harmonious utilization of the existing technical and material resources within the discipline for the support of the lectures and the practical training, to achieve an effective management of teaching time by using a course pattern to support understanding and preservation of the new information;
- Supporting educational material with clinical examples, complex eloquent case studies, practical demonstrations, multi-media presentations;
- Improving the didactic practice approaches by applying an active-participatory strategy which involves the student in the teaching-learning process, solving hypothetical clinical situations;
- Keep up-to-date the curriculum content, clinical traineeship and provision of the information, in line with periodontology novelties and technology capabilities;
- Gathering information and data collection, so that, together with the members of the discipline, to issue a practical guide, as well as a book volume in the specialty of Periodontology for the systemically affected patient, for the sixth year students in Romanian, English and French teaching programs;
- Active participation in national and international scientific events;
- Diversification of assessment techniques based on student performance and skills, periodic evaluation of the student tasks in order to become familiar with the methods of final examination and evaluation;
- Collaboration with other national centers to facilitate student mobility;
- Creating a strong teamwork within the Periodontology discipline for interested and skilled students, in which to be able to carry out short-term research programs.

The student-centered teaching method will be continued with the future involvement of the student in practical virtual cases that needs the application of specific knowledge, in order

to interconnect the practical and theoretical data from lectures and applied activities. The postgraduate programs with master students and resident doctors in orthodontics, endodontics, surgery, prosthetics and pedodontics will continue during the periodontology module as well, to stimulate the young doctors to discover that the challenging aspects of the research activity are based on creativity and on solid knowledge.

Residents

Regarding the residents - who have already chosen their professional direction and thus are more focused on certain topics, the teaching process has two aspects: one aims at consolidating the basic medical knowledge necessary to make an accurate diagnosis and a decision-making process in the best interest of the patient, and the other aims to develop and improve the theoretical and practical skills necessary for the complex treatment of periodontal disease. The close collaboration with the residents gives me a clearer image about their need to form and evolve, the relationship being bidirectional because through their enthusiasm and curiosity they can bring news and updates in diagnostic methods or instrumental techniques.

I am considering further extension of the involvement of the specialized residents in research projects and in the elaboration of scientific articles. Although conceptually their contribution may not be major, their involvement in the clinical examination of patients in the configuration of databases will give them a more accurate view of the links between scientific research and the practice of dentistry. This approach on resident training represents the continuation of activities already carried out on the occasion of the internal Grant project carried out in interdisciplinary collaboration with the discipline of nephrology, in which during 2013-2014, year 2 residents in the periodontology specialty were calibrated as examiners and involved in the screening of a group of 400 haemodialysis patients from two dialysis centres in Iasi.

I found that this assertive approach on resident training opens up opportunities for some of them to continue their training within the doctoral program, and some even to aim for an academic position.

Corresponding to the academic activities regarding resident doctors, the main future development directions are as follows:

- Enhancing the training of resident doctors by teaching up-to-date, applicable medical content using updated course materials;
- Organizing clinical presentations and presentations of research in the existing literature on specific designated topics, with the participation of residents, specialists and primary physicians in order to stimulate debate and discussion;
- Encourage the active implication of residents in the learning process with accent on supervised practical activities, in accord to the resident log book;
- Teach the importance of participating in multidisciplinary teams in approaching complex cases and organize sessions of case presentation with multidisciplinary participation;
- Implementing a rigorous knowledge evaluation system regarding theoretic and practical abilities;
- Encouraging and guiding the active participation of residents in research projects, national and international scientific events, as well as application for scholarships;
- Encourage learning by experience exchange in national and international centers.

Doctoral students

Coordination of doctoral students is both a mission and a task of great importance. During my career I had the privilege to collaborate on various scientific projects with several doctoral coordinators: Prof. Radu Vataman, Prof. St. Lăcătușu, Prof. Silvia Mârțu. Under their competent guidance, numerous clinical and laboratory studies were carried out, which substantiated a considerable number of doctoral theses and resulted in dozens of articles published in prestigious journals ISI and BDI indexed, as well as lectures and oral communications or posters presented at prestigious world-class scientific events such as the EUROPERIO series of congresses, where they were highly appreciated. Also, my constant participation in most doctoral guidance commissions for doctoral theses coordinated by Prof. PhD Silvia Mârțu, trained me for what it means to coordinate a doctoral scientific research, what it means to mentor a doctoral student from shaping the research idea to its completion by defending the thesis, going through the configuration of the studies, then the centralization and statistical processing of the databases and finally the pertinent interpretation of the results.

Regarding the academinc activities involving doctoral students, the main future development directions are as follows:

- Selecting the most capable students and resident doctors showing interest in learning and performing scientific research, for doctoral studies fellowship;
- Establish specific areas of recent interest and development in the field of Oral and Maxillofacial surgery that would be suitable for doctoral studies, in relation to the existing and ongoing research subjects;
- Teach doctorate students the newest information regarding the research topic and the practical means of achieving specific objectives, as a result of academic and clinical experience;
- Encourage doctorate students to participate in multidisciplinary teams in order to broaden the spectre of ideas and find new technological solutions;
- Follow opportunities of interdisciplinary and inter-university collaborations in order to facilitate access to information, technology and experience exchange;
-Encourage doctorate students to make their work vissible through publishing articles, presenting their work as oral presentations or posters during various congresses and symposiums.

SECTION III

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