GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

CURRENT TRENDS IN PERIODONTAL DIAGNOSIS AND THERAPY - BETWEEN CLINICAL AND EXPERIMENTAL APPROACH

- HABILITATION THESIS -

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ABREVIATIONS

ATP - adenosine triphosphate
OXPHOS - oxidative phosphorylation
mtDNA - mitochondrial DNA
ROS - reactive oxygen species
MS - metabolic syndrome
T2DM - type II diabetes mellitus
NADH - reduced nicotinamide adenine dinucleotide
FADH2 - reduced flavin adenine dinucleotide
TCA - tricarboxylic acid
ETC - electron transport chain
UQ - ubiquinone
UQH2 - ubiquinol
Q - semi-quinone radical ion Q-
Hs - heavy strand
Ls - light strand
NCR - non-coding region
D – loop displacement loop
OH - origin of heavy-strand synthesis
HSP - heavy-strand promoter
LSP - light-strand promoter
TLR9 - toll-like receptor 9
TOM - translocase of the outer mitochondrial membrane
TIM - translocase of the inner mitochondrial membrane
Mfn - mitofusins
OPA1 - optic atrophy 1 protein
Drp1 - dynamin-related protein 1
Fis1 - fission protein
PGC - 1αproliferator-activated receptor gamma coactivator-1α
NRF - nuclear respiratory factor
TFA mt - mitochondrial transcription factor
AMPKAMP - activated protein kinase
SIRT1 - silent information regulator 1
O2•− - superoxide radical anions
H2O2 - hydrogen peroxide
MAO - monoamine oxidase
1O2 - singlet oxygen
OH• - hydroxyl radical
HOO• - hydroperoxyl radical
NO• - nitric oxide
RNS - reactive nitrogen species
NOS - nitric oxide synthases
GLUT4 - glucose transporter 4
EXT - exostosin
NAC - N-acetylcysteine
COQ10 - coenzyme Q10
MitoQ - mitoquinone
MitoE - mitovitamin E
TPP+ - triphenylphosphonium cation
ACC - artificial cariogenic challenge
SEM - electron microscopic
EDX - X-ray spectroscopy
MMSE - Mini-Mental State Examination
MCI - moderate cognitive impairment
AD - Alzheimer's disease
DM - Diabetes mellitus
SRP - scaling and root planning
HbA1c - hemoglobin A1c
MMPs - matrix metalloproteinases
SDD - subantimicrobial doses
FDA - Food and Drug Administration
PD - Probing depth
CAL - clinical periodontal attachment loss
BOP - bleeding on probing
IFCC - International Federation of Clinical Chemistry
BMI - body mass index
GI - gingival index

GBI - gingival bleeding index
FEM - finite element method
ECMs - extracellular matrix proteins
PMNLs - polymorphonuclear leukocytes
TNF α - tumor necrosis factor α
GMCSF - granulocyte-macrophage colony-stimulating factor
GFC - gingival crevicular fluid
ROS - reactive oxygen species
MPO - release of myeloperoxidase
CHX - chlorhexidine
GECs - gingival epithelial cells
MCPIP-1 - monocyte chemoattractant protein-1-induced protein
MALT-1 - mucosa-associated lymphoid tissue lymphoma translocation protein
EMS - electromagnetic spectrum
NIR - near-infrared
HEV - High-energy visible
LASER - Light Amplification via the Stimulated Emission of Radiation.
Nd:YAG - Yttrium Aluminum Garnet Laser
PDT - photodynamic treatment
PS – photosensitizer
AS - absorption spectrum
LLLT - low-level light therapy
PBMT - photobiomodu- lation therapy
TMD - temporomandibular disorder
AGEs - glycation end-products
SRP - Scaling and root planing
aPDT - Antimicrobial photodynamic therapy

ABSTRACT OF THE THESIS

An academic career requires the fusion between the profession of scholar mentor, researcher and medical practitioner and stands on the top of the educational system and its success is established on perseverance and self-indulgence, responsiveness and openness to new ideas and concepts. Assiduity and complaisance, character and critical reflection are also required. Self-determination and implementation of professional targets and standards in academic careers is essential and mandatory in order to ensure the continuous increase in quality of the educational process and of the scientific and medical practice results. Education is a necessity in the development of the individual and, implicitly, of society. Each of us is encouraged and helped to make use of the opportunities to learn offered throughout life. Once we become academics, we must possess the necessary knowledge and skills, have certain individual characteristics suitable for the profession, as well as the motivation and the right professional perspectives. Some of these we are born with, such as oratory and pedagogical talent, others must be learned, but it is very important for both to be practiced and improved. This is possible through hard, continuous work as well as through the reference models we have.

Present-day distinguished academic careers meet standards and highlight awareness of the necessity for continuous formation, the integration of modern techniques into teaching and dentistry practice. Formerly, these principles match, they work together for an increase in the educational process quality and its results, the improvement of scientific performance and the success in the dentistry career. The university career of each of us has and must have a compelling impact upon the gross academic community because it has to combine accordingly a large number of accomplishments, know-how and qualities, such as: solid and constantly updated scientific acquaintance, availability and pleasure to communicate and teamwork, along with the desire to be part of a team. All of these cast the ability to create and coordinate functional structures, based on the ability to identify and motivate human resources through their own standard.

The habilitation thesis in conducting PhD studies compiles my entire professional, academic and scientific activity after the completion of doctoral research. It is structured according to the criteria recommended and approved by CNATDCU, in three major sections. In this respect, this paper highlights both the overall picture and the detailed overview of the main concerns and objectives of my academic career so far. Based on personal experience in domains of interest, I will delineate the management of the ongoing research projects, along with the studies and implementation opportunities that they open.

In the midst of the cardinal concerns I have had since the beginning of my academic career there is the concern to address new areas of research with direct implications in Dentistry, especially in periodontal practice and, particularly, for the benefit of patients. It is now unfolded in a series of future projects which are described in the corresponding section of the thesis. It also accommodates detailed descriptions of the future research projects that I want to follow in the coming years.

In this thesis I outline my research, didactic and medical work carried out since 2016, year which corresponds to the finalization and presentation of my PhD thesis, with the title " Studies of Periodontal Biodynamics during Orthodontic Therapy Associated with Periodontal Disease" under the coordination of Prof. Dr. Silvia Mârţu.

The content of the thesis is divided into three main sections: SECTION I, detailing my academic, medical and research work; SECTION II, which traces on the main results of my research activity, materialized by publication of ISI rated articles with impact factor as a point of continuation of the research started but also for future projects; and SECTION III, which contains the bibliographic references.

SECTION I contains the results of my entire professional, scientific and academic work in three chapters and summarizes the main personal contributions in the field of periodontology. There are the three distinctive domains of my academic activity to which I have devoted almost my entire career. The first one refers to my entire medical practice. The second and the most extensive one regards my academic career development. Finally, the third one approaches the research field of my activity.

By bringing to light personal motivation in choosing research topics and the way ahead and implementing them, I intend to prove both the capacity to initiate and develop personal research projects, as well as my teamworking abilities. The ultimate goal of the efforts and work carried out by me and the teams I co-ordinate is the patient's benefit in everything that means advanced technology and healthcare professionals trained to give special, professional and innovative care for the periodontal patients.

The performance achieved in the dental medicine career is based on the opportunities opened by the continuous medical training courses that I have followed in the country and abroad.

The scientific research I have had so far has materialized in book publications, articles, and communications at congresses. All this hides behind the hard teamwork and passion for what we do. The dissemination of the results of the scientific research would not have been possible without the help of the medical school trainers who coordinated my medical and academic career and from whom I learned this specialty in the field of periodontology.

I am convinced that the academic level I have achieved ensures a great international visibility and, directly, the prestige of the university I represent.

The theme chosen for the doctoral study and its manners and opportunities for continuity have opened both the way to do research in the field of periodontology and new research directions. During the doctoral study, I learned and perfected my research skills so necessary for my future career. I have been familiar with the early, clinical and radiological diagnostic techniques of these pathologies. My research in this direction focused on highlighting the modern means of diagnosis and treatment in the pathology of all diseases regarding periodontology, from prevention, to diagnosis, treatment and post-treatment care.

In the second part - SECTION II - I grouped my scientific and professional achievements in order to certificate my future research projects which are detailed in this section.

The assessment of the Dentistry and, especially of periodontal patients in both elective and emergency situations, the research in training medical teams and their multidisciplinary approach are motivated by the passion I have always had for this domain. In order to be able to perform in my field, it is necessary to put feelings into what you do and to constantly update your knowledge.

These branches and directions of periodontology academic activity have also benefited from the current advances in related fields: radiology, computing, geriatrics and bioengineering.

The outcomes of my current academic practice are mirrored in materials published in ISI-rated journals with impact factor. I emphasize this section's material proves my interest in researching new methods of early diagnosis and staging of these conditions. In this section, the results of my personal research on the specific management of periodontal patients are also represented, correlated with the main areas of research interest. The section is subdivided into three subchapters. The first highlights my personal research related to etiopathogenic risk factors of periodontal pathology.

The second sub-chapter highlights the results obtained in the research we carried out on modern diagnostic methods and the management of patients with periodontal pathology.

The third subchapter contains the results of my research in terms of modern research directions in periodontology.

Finally, this section puts into perspective my personal achievements in the niche areas represented by the applicability of results in research fields related to my areas of interest.

The final chapter of the thesis - SECTION III - represents the scientific foundation of my current research activity and the basis for my future study plans and projects. This section contains all the bibliographic references on which I have built my current knowledge in the areas of interest and the support of their permanent update.

REZUMATUL TEZEI

O carieră academică necesită fuziunea între profesia de mentor, cercetător și medic, fiind în vârful sistemului educațional, iar succesul ei se stabilește prin perseverență și auto-exigență, receptivitate și deschidere către idei și concepte noi. De asemenea, sunt necesare flexibilitate, dinamism și reflecție critică. Autodeterminarea și implementarea obiectivelor și standardelor profesionale în cariera academică este esențială și obligatorie pentru a asigura creșterea continuă a calității procesului de învățământ și a rezultatelor practicii științifice și medicale. Educaţia este o necesitate în dezvoltarea individului şi, implicit a societăţii. Fiecare dintre noi este încurajat şi ajutat să se folosească de posibilităţile de a învăţa oferite pe parcursul vieţii. Odată ajunși cadre universitare trebuie să deţinem cunoştinţele şi deprinderile necesare, să avem anumite caracteristici individuale adecvate profesiei cât și motivaţia şi perspectivele profesionale potrivite. Cu unele dintre acestea ne naștem, cum ar fi talentul de orator și cel pedagogic, altele trebuie învățate dar, ambele este foarte important de a fi exersate și îmbunătățite. Acest lucru este posibil printr-o muncă asiduă, continuă cât și prin modelele de referință pe care le avem.

În prezent carierele academice îndeplinesc standarde și evidențiază conștientizarea necesității formării continue, a integrării tehnicilor moderne în predare și în practica stomatologică. Aceste principii sunt interconectate între ele în vederea creșterii calității procesului educațional și rezultatele acestuia, îmbunătățirii performanței științifice și succesul în carieră.

Cariera universitară a fiecăruia dintre noi are și trebuie să aibă un impact convingător asupra comunității academice deoarece trebuie să combine în consecință un număr mare de realizări și calități, precum: cunoștințe științifice solide și permanent actualizate, disponibilitate și capacitatea de comunicare și de a putea face parte dintr-o echipă.

Teza de abilitare întocmește întreaga mea activitate profesională, academică și științifică după finalizarea cercetării doctorale. Este structurată după criteriile recomandate și aprobate de CNATDCU, în trei mari secțiuni. În acest sens, prezenta lucrare evidențiază atât imaginea de ansamblu, cât și imaginea detaliată a principalelor preocupări și obiective ale carierei mele academice de până acum. Având în vedere experiența personală în multiple domenii de interes, voi delimita managementul proiectelor de cercetare în derulare, împreună cu studiile și oportunitățile de implementare pe care acestea le deschid.

În mijlocul preocupărilor cardinale pe care le am încă de la începutul carierei mele academice se află capacitatea de a aborda noi domenii de cercetare cu implicații directe în stomatologie și mai ales în practica parodontală și, în special, în beneficiul pacienților. Acestea sunt descrise în secțiunea corespunzătoare a tezei. De asemenea, teza prezintă descrieri detaliate ale viitoarelor proiecte de cercetare pe care le voi întreprinde.

În această teză conturez activitatea de cercetare, didactică și medicală desfășurată începând cu anul 2016, an care corespunde finalizării și prezentării tezei mele de doctorat, cu titlul „Studii de biodinamică parodontală în terapia ortodontică a bolii parodontale” sub coordonarea Prof. Dr. Silvia Mârţu.

Conținutul tezei este împărțit în trei secțiuni principale: SECȚIUNEA I care detaliază activitatea mea academică, medicală și de cercetare, SECȚIUNEA II, care urmărește principalele rezultate ale activității mele de cercetare, concretizate prin publicarea unor articole cotate ISI cu factor de impact, ca punct de continuare a cercetării începute dar și pentru proiecte viitoare și SECȚIUNEA III care conține referințele bibliografice.

SECȚIUNEA I cuprinde rezultatele întregii mele activități profesionale, științifice și academice în trei capitole și rezumă principalele contribuții personale în domeniul parodontologiei. Sunt descrise trei domenii distincte ale activității mele academice cărora le-am dedicat aproape întreaga mea carieră. Primul se referă la practica medicală, al doilea și cel mai extins se referă la dezvoltarea carierei mele academice, iar al treilea abordează domeniul de cercetare al activității mele.

Prin evidențierea motivației personale în alegerea temelor de cercetare, a modalității de efectuare și prin implementarea acestora intenționez să demonstrez atât capacitatea de a iniția și dezvolta proiecte personale de cercetare, cât și abilitățile mele de lucru în echipă. Scopul final al eforturilor și muncii depuse de mine și de echipele pe care le coordonez este beneficiul pacientului în tot ceea ce înseamnă tehnologie avansată și profesioniști din domeniul sănătății pregătiți pentru a oferi îngrijiri speciale, profesionale și inovatoare pacienților cu patologie parodontală.

Performanța realizată în cariera stomatologică se bazează pe oportunitățile deschise de cursurile de formare medicală continuă pe care le-am urmat în țară și în străinătate.

Cercetările științifice pe care le-am avut până acum s-au concretizat în publicații de cărți, articole și comunicări la congrese. Toate acestea se ascund în spatele muncii de echipă și pasiunii pentru ceea ce facem. Diseminarea rezultatelor cercetării științifice nu ar fi fost posibilă fără ajutorul formatorilor școlii de medicină care mi-au coordonat cariera medicală și academică și de la care am învățat această specialitate în domeniul parodontologiei.

Sunt convins că nivelul academic pe care l-am atins asigură o mare vizibilitate internațională și, în mod direct, prestigiul universității pe care o reprezint.

Tema aleasă pentru studiul de doctorat și manierele și oportunitățile sale de continuitate au deschis calea cercetării în domeniul parodontologiei și au deschis noi direcții de cercetare. În cadrul studiului de doctorat mi-am perfecționat și perfecționat abilitățile de cercetare atât de necesare carierei mele viitoare, m-am familiarizat cu tehnicile de diagnostic precoce, clinice, preclinice și radiologice ale acestei patologii.

Cercetările mele în această direcție s-au concentrat pe evidențierea mijloacelor moderne de diagnostic și tratament în patologia paradontală, de la prevenție, până la diagnostic, tratament și îngrijire post-tratament.

În partea a doua - SECȚIUNEA II - am grupat realizările mele științifice și profesionale pentru a-mi certifica viitoarele proiecte de cercetare care sunt detaliate în această secțiune.

Evaluarea în stomatologie și, mai ales a pacienților parodontali atât în situații elective cât și în situații de urgență, cercetarea în formarea echipelor medicale și abordarea multidisciplinară a acestora sunt motivate de pasiunea pe care am avut-o întotdeauna pentru acest domeniu. Pentru

a putea performa în domeniul meu, este necesar să te implici sufletește în ceea ce faci și să-ți actualizezi constant cunoștințele. Aceste ramuri și direcții ale activității academice au beneficiat și de progresele actuale în domenii conexe: radiologie, informatică, și bioinginerie.

Rezultatele practicii mele academice actuale sunt reflectate în materiale publicate în reviste clasificate ISI cu factor de impact. Subliniez că materialul acestei secțiuni dovedește interesul meu pentru cercetarea unor noi metode de diagnosticare precoce și stadializare a acestor afecțiuni.

În această secțiune sunt prezentate și rezultatele cercetărilor personale privind managementul specific al pacienților parodontali, corelate cu principalele domenii de interes de cercetare. Este subdivizată în trei subcapitole. Prima dintre acestea evidențiază cercetările personale legate de factorii de risc etiopatogeni ai patologiei parodontale. Al doilea subcapitol evidențiază rezultatele obținute în cercetarea asupra metodelor moderne de diagnostic și al managementului pacienților cu patologie parodontală. Al treilea subcapitol conține rezultatele cercetării mele în ceea ce privește direcțiile moderne de cercetare în parodontologie.

Capitolul final al tezei - SECȚIUNEA a III-a - reprezintă fundamentul științific al activității mele actuale de cercetare și baza pentru planurile și proiectele mele de studiu viitoare. Această secțiune conține toate referințele bibliografice pe care mi-am construit cunoștințele actuale în domeniile de interes și suportul actualizării permanente a acestora.

SECTION I
SCIENTIFIC ACHIEVEMENTS FROM THE POSTDOCTORAL PERIOD

Overview of the academic, scientific and professional achievements

The concept of a career as the inevitable result of combining one's passion with available work is misleading. Clarity on my professional path came when I finally committed to the developmental process of connecting what I know about myself with what I know about my three primary fields of work (clinical stomatology, stomatology education and dentistry research). From their earliest days in school, students are exposed to the allure of a career in academia. Having the opportunity to practice medicine and educate, two of the most beautiful and honourable professions, is a blessing. To achieve this level of competence, however, requires a lifetime of dedication, hard work, fulfilment, sacrifice, and responsibility.

I am fortunate enough to work at the same institution where Professor Leon Scully taught his first anatomy class 141 years ago. The weight of history not only validates us, but also compels us. The integration of higher education with European standards and norms is just one indicator that we live in a dynamic society characterised by fundamental shifts towards modernity. Times Higher Education's Emerging Economies University Rankings placed the University of Medicine and Pharmacy "Grigore T. Popa" Iasi at the top of the list for Romania and in the top half of the universities studied worldwide.

Career maturity, or the ability to make sound decisions, is a humanist concept, defined as the acquisition of sufficient vocational knowledge, self-knowledge, and decision-making knowledge via deliberate research. As I reflect on my development thus far, I have come to terms with the struggles one must overcome and the reality that reaching this level of professional development should be an ongoing effort.

Having had the opportunity to develop professionally in a multicultural, multifunctional environment that fostered the growth of critical abilities, in accordance with a consistent set of values upon which I rely (high moral and professional conduct, mutual respect, sensitivity to human diversity, and cultural anchors), has allowed me to successfully straddle the three main areas of teaching, practise and research.

My career development has both a professional and, especially, a humanistic side. These sides are the cumulative, objective and qualitative result of personal life experiences. All the efforts made to achieve a high level of performance together with academic and medical recognition are crowned with success by the results obtained in current practice.

The research perspective stimulated me throughout my university career and focused on the medical and surgical activity of periodontology and the issues related to it, topics with major socio-economic impact. The research activity that I have carried out up to this moment is mostly addressed to the main domain of interest: periodontology.

The didactic activity, supported by the personal medical results, I oriented towards the same major research direction. I consider our role as medical school creators fundamental, in order to stimulate, encourage and train future doctors in the exploration of this theme.

Professional progress

After 14 years of medical practice and the academical experience, I can say that the results of my career are integrated into the complexity of the requirements and demands of the medical field and, first of all, of the research.

1999-2003	HIGH-SCHOOL DIPLOMA, NO. 0312390/16.VII.2003, MATHEMATICS-PHYSICS PROFILE "EUDOXIU HURMUZACHI",RADAUȚI-SUCEAVA
2003-2009	DOCTOR - DENTIST, BACHELOR'S DEGREE NO. 488/ 21.XI.2009 FACULTY OF DENTAL MEDICINE, UNIVERSITY OF MEDICINE AND PHARMACY "GRIGORE T. POPA", IAȘI
2011	CERTIFICATE OF FURTHER EDUCATION IN IMPLANTOLOGY ISSUED BY THE ROMANIAN MINISTRY OF HEALTH ON 12.IV.2011
2011	CERTIFICATE OF FURTHER EDUCATION IN PEDODONTICS IN 12.IV.2011
2012	CERTIFICATE OF FURTHER EDUCATION IN THERAPEUTIC AND SURGICAL USE OF LASERS IN DENTISTRY 27.IX.2012
2016	SPECIALIST IN PERIODONTOLOGY , CONFIRMED BY THE ORDER OF THE MINISTER OF HEALTH NO.1 /2016
2021	CONSULTANT/SENIOR SPECIALIST IN PERIODONTOLOGY CONFIRMED BY THE ORDER OF THE MINISTER OF HEALTH NO.1749 /03.IX.2021

During all this period I gained a rich experience in the management of the student series, and of the activities carried out with the residents but also in terms of my main goal - ensuring and maintaining the anatomy and physiology of the dental health of my patients. The purpose of this sustained effort is to give them the joy of a better life, managing to achieve and, for as long as they could, maintain their dental health.

In addition to my lifelong pursuit of dental excellence - which I demonstrate by regularly attending both national and international scientific conferences and post-graduate courses, I have a wide range of other interests, including those in implantology and dental lasers.

The main future objectives of my professional career are related to the continuation of research in the major directions related to periodontology; research of the etiopathogenic factors, treatment, management of the periodontal disease and future maintenance of the gingival health.

At the same time, the efforts made by the medical team I coordinate in the complex study of periodontology, are about to anticipate that the results which will materialize through reference publications and would be an extremely valuable working tool in assessing the socio-economic, demographic impact and in creating a management protocol, so necessary for these patients.

In the medium term, I am considering applying for a multi-institutional grant regarding the medium and long-term socio-economic impact of the increase in risk factors, as well as writing a family guide addressed to both patients and young doctors.

I intend that all these approaches and efforts co-interest as many colleagues as possible in this public health problem, with the aim of coagulation of elite, multidisciplinary medical centers and teams that will also be medical school trainers in this direction. Without such collaborations and the related human resource, our research in the mentioned fields could not materialize.

Hard work was and will be the foundation of my academic career, along with the awareness of the imperative need for research and the opportunities provided by the training internships carried out in the country and abroad. This step towards exchanges of experience and research with centers of excellence from abroad, in co-domains of interest, is essential to keep us in touch with current trends and the latest discoveries in the field.

I consider it a great opportunity given to me by the possibility that I have to correlate the three great sides of my career - teaching, dentistry and research - around the main theme of interest, which brings together all the others. This is about the desire to preserve oral health and prevent periodontal pathology, which my practical work tries to paint in the warmest colors.

Academic activity and career overview

I started my university career in 2011 at the "Grigore T. Popa" University of Medicine and Pharmacy of Iasi, Faculty of Dental Medicine, Department of Periodontology. Since 2013, I have continued as a university assistant in the Department of Implantology, Removable Restorations, Technology, Discipline of Dental Prosthetics Technology, within the same faculty.

Between 2011-2012, I followed the master's course "Periodontal rehabilitation through complex conventional and surgical techniques", within our university.

Medical practice for over 14 years, together with a rich academic experience, have given me a wealth of experience that I have mirrored in and on the scientific research side. The activity as vice-dean of the Faculty of Dental Medicine within the University of Medicine and Pharmacy "Grigore T. Popa" of Iasi, represents a significant part of my activity up to now. The activity carried out in these directions, under the coordination of some elite personalities and in nationally and internationally recognized centers, have formed and channeled me in my career so far.

I have enriched my personal experience, knowledge and capabilities by attending postgraduate training courses in co-domains of interest. All this materialized by obtaining professional certificates that allow me to practice maneuvers and techniques that are so necessary and useful in my current practical and research activity. These certificates are:

- Master's degree "Oral rehabilitation on implants". - 2011;
- Specialization and Accreditation Course - Certificate of Active Participation for the Chao Pinhole Surgical Technique, delivered by Dr. John Chao, Alhambra, CA, United States of America

- Training/documentation internship – Western University of Health Sciences, CA, United States of America.
- 2nd European Federation of Periodontology Master Clinic, Valletta, Malta.
- 1st European Federation of Periodontology Master Clinic, Paris, France.
- "Laser Periodontology Training" course held by Dr. Alfredo Aragues (World Clinical Laser Institute), Bucharest, Romania.
- Public Services Management Course, Faculty of Economics and Business Administration, "Alexandru Ioan Cuza" University, Iasi, Romania

The practical usefulness of these professional skills that I have thanks to obtaining the certificates was also reflected on my scientific research in the field, where it materialized through the publication of ISI-rated articles with an impact factor. The most relevant materials obtained refer to the study of risk factors, diagnostic methods and establishing the prognosis as well as the management of periodontal pathology.The current professional level we have reached would not have been possible without these professional skills.

To engage students in developing employability skills, knowledge and experiences, academics and university professionals implement a range of approaches and strategies, for example work-integrated learning (WIL) and co-curricular experiences. One of the key elements to approach the development of graduate employability includes teaching students about career options, tools, and possibilities.

Career development learning (CDL) centralises purposeful strategies and interventions to 'help individuals self-actualise, transition to the labour market, make the best use of their skills and knowledge and live happy and fulfilled lives. CDL engages students in self-assessment to appraise their priorities and relate situated learning to their future career or profession. Examples of CDL might include identifying personal skills, knowledge and interests and evaluating priorities, developing strategies for searching career opportunities, using tools for networking, showcasing strengths and creating short/medium term career action plans. As a self-managed process, CDL aligns with educational approaches that employ reflection, critical thinking and application of knowledge and skills to make meaning connections to work and career.

In the higher education context today, a reflexive approach to employability is necessary to build graduates' capabilities and skills for navigating labour market complexity. Graduates who understand and apply the wider value of their qualifications through transferable skills and knowledge, will be better equipped to succeed in a dynamic economic conditions and distributed workplace environments.

Most universities offer career services, where qualified practitioners support students in career related decisions and goals. These centres work closely alongside or within faculties to provide bespoke advice and resources, a source of collaborative expertise and relationship with students, academics and industry and as a vice dean of the Faculty of Dental Medicine I intend to work on these direction. CDL has not traditionally been part of academic positions, nor is it typically incorporated into formal education or accreditation of higher education programs. Historically, it has not been recognised in workload tools or probation or promotion processes. It is no wonder then, that academics have identified barriers and challenges to implementing CDL.

Without dedicating myself wholeheartedly to professional development throughout college, I would not have been able to handle all these responsibilities I was given. I committed to keeping abreast of the subject of periodontology by attending the annual Congresses hosted by the Romanian Society of Periodontology and UNAS, as well as the Europerio Congresses hosted by the European Federation of Periodontology. As a whole, the time and effort I've put in as a member of both the discipline team and the faculty department have been recognised in a number of different ways.

2011-2013	ASSISTANT OF RESEARCH – DEPARTMENT OF PERIODONTOLOGY , FACULTY OF DENTAL MEDICINE, "GRIGORE T. POPA" U.M.PH. IAȘI
2013-2018	ASSISTANT PROFESSOR – DEPARTMENT OF DENTAL TECHNOLOGY , FACULTY OF DENTAL MEDICINE, "GRIGORE T. POPA" U.M.PH. IAȘI
2018-2022	LECTURER - DEPARTMENT OF PERIODONTOLOGY , FACULTY OF DENTAL MEDICINE, "GRIGORE T. POPA" U.M.PH. IAȘI
2022-present	ASSOCIATE PROFESSOR - DEPARTMENT OF PERIODONTOLOGY, FACULTY OF DENTAL MEDICINE, "GRIGORE T. POPA" U.M.PH. IAȘI
2011-2016	PhD - Doctor in Dental Medicine – confirmed by the Order of the Minister of Education and Research no. 3148/30.01.2017; thesis "Studies of periodontal biodynamics DURING orthodontic therapy associated with periodontal disease" scientific coordinator Prof. Dr.Martu Silvia

Research projects and scientific activities

My research projects aim at the same common goal as my career - ensuring and maintaining dental health, related to the prevention and management of periodontal pathology. Until now, I have published books and articles on topics of interest and have communicated the results obtained at relevant congresses.

Studies conducted on groups of patients have a strong professional impact due to their statistically significant number and predefined and precise selection criteria.

The coordination of several medical teams, which I achieved during the years of medical experience and the collaboration with other reference centers in the field within the country, made my training as a researcher possible and provided me with a vast experience in the field.

Above, in the academic section of this brief review, I reflected on how the international scientific community has structured the field of periodontology, and how I have been able to actively contribute to shaping the discipline in our Faculty by creating relevant courses and practical activities for students in their final years. The novelty with which my research activity comes to the fore is given by its immediate clinical integration and the adaptability that I and my team have shown. Thus, every study we undertook was conducted and restructured according to real-time feedback and information received from subjects, which we interpreted and analysed.

The careful follow-up of patients, both during the hospitalization period and at a distance, makes it possible to obtain a valuable database with a strong potential in future studies.

I consider that a mandatory and inherent added value of my research activity is the winning and implementation of Grant-type study projects, national and international.

Synthesis of the PhD thesis

The doctoral study that I completed was titled "Studies of periodontal biodynamics in orthodontic therapy for periodontal disease" under the coordination of Prof. Dr. Silvia Mârţu.

This study practically opened the side of personal research oriented towards periodontal pathology. A special personal interest in this study is related to the management and prevention of this pathology as well as the last generation minimally invasive treatment techniques.

Starting from the results and premises of the doctoral study, the way is opened for me to study the possibilities of preserving oral health for as long as possible, of preventing the periodontal pathology of the patients I treat and advise in this field, as well as, possibly, to treat it with a quality/surgical stress ratio as high as possible.

International visibility. In all my career years, I constantly strived to increase my international visibility by actively participating in international congresses and publishing articles in academic journals with high/very good impact factor.

In order to increase my teaching abilities I took part as an expert in the educational project won through compention InterMedIS 6.0 and InterMedIS 4.0. I also was a member of the team in the project supported by the French University Agency (AUF).

A H-index of 19 and a total of 692 citations, according to data from the Web of Science website, proves that our research is earning international and national recognition. I have also tried to increase my international visibility by using hosting services such as SlideShare to disseminate our research results. To the same end I use the ORCID platform that provides a persistent identity for humans for a *better international visibility.*

My scientometric parameters are synthetized bellow:

- *HIRSCH INDEX(CLARIVATE ANALYTICS):* ***19***
- *NUMBER OF PUBLICATIONS IN CLARIVATE ANALYTICS DATABASE:* **102**
- *CUMULATIVE IMPACT FACTOR (main author* since last promotion*):* **57.4**
- *TOTAL NUMBER OF CITATIONS WITHOUT SELF-CITATIONS (CLARIVATE ANALYTICS):* **448**
- *AVERAGE CITATION PER ITEM:* **6.78**
- *HIRSCH INDEX (GOOGLE SCHOLAR):* **17**
- EDUCATIONAL AND SCIENTIFIC PROJECTS*:* **3**
- *ORCID:* 0000-0003-0017-9672

CHAPTER 1:
ALL ROADS LEAD TO PERIODONTAL DISEASE. CURRENT KNOWLEDGE ON ASSOCIATED RISK FACTORS OF PERIODONTITIS.

1.1. State of the Art

Modern dentistry and anything related to it, is only a few centuries old. Unfortunately, the need for oral care is as old as humanity itself. Within this context, periodontal diseases are considered as old as the history of mankind (Yilmaz S, et al.,1994). Throughout the endless millennia, techniques have been discovered, tried, and forgotten. It was until the 1800's that we began truly understanding oral health. First important step was the definition of periodontal disease, given by John T. Riggs defined who defined it as a "suppurative inflammation of the gums." This term refers to conditions that generate significant amounts of pus. Gum disease certainly matches this description in its later stages (Shklar et al., 2002).

Historically, the etiology of periodontal diseases has focused on bacterial plaque, microbial by-products, and the host immune response. Although recent studies have suggested a role for environmental (Genco 1996), behavioral (Grossi et al., 1994; Grossi et al., 1995), and genetic (Kornman K et al., 1997) risk factors in periodontal disease progression. Most, if not all, forms of periodontitis should be viewed as infectious diseases. Bacteria are the primary etiologic factor of periodontal diseases, however, recent evidence also lists yeast and herpesviruses as putative pathogens (Ontreras and Slots 2000). Meanwhile, our understanding of the pathogenic process has been hindered by the fact that it is usually the result of a polymicrobial infection including indigenous organisms with little pathogenic potential (Guthmiller and Novak 2002).

It is now generally agreed that almost all forms of periodontal disease occur as a result of mixed microbial infections within which specific groups of pathogenic bacteria coexist (Ruby et al., 2002; Blandino et al., 2007; Pussinen et al., 2007). Evidence is reviewed on the potential roles of modifiable and nonmodifiable risk factors associated with periodontal disease. An understanding of risk factors is essential for clinical practice.

The ancient Native Americans ate a diet mostly of corn (maize), beans, squash, fish, and game, as well as fresh fruit and nuts. Their high-fiber diet helped keep their teeth and gums healthy. The harmful bacteria in our mouths need plenty of sugar and starch to multiply. Foods high in fiber have the benefit of scrubbing our teeth as we eat them. Native Americans cleaned their teeth by using chewsticks and chewing on fresh herbs to cleanse their teeth and gums. Chewsticks were twigs that had two uses: one end was frayed by a rock and used for brushing, while the other end was sharpened and used as a tooth pick. Native Americans would chew on the frayed end to clean debris from their teeth.

In addition to chewsticks, early Native Americans would also chew on pine needles to clean debris from their teeth. They also chewed fresh herbs like sage, cucacua and mint to freshen their breath (Goldberg et al., 1976).

The first pilgrims to colonize America were a completely different story. The Pilgrims' journey from England to America took 66 days by ship. In order to survive the lengthy journey, they stocked their ships with preserved food like salted dried meat, dried fruit and hardtack – a dried biscuit that is very similar to a saltine cracker. All of these foods are terrible for teeth, particularly dried fruit and hardtack, which easily sticks to teeth and provides fuel to bad bacteria that cause cavities. What the pilgrims drank on their voyage may have been even worse. Because water distillation was not perfected yet, sailors of the time didn't drink a lot of water because it was unclean and could easily lead to sickness and death. This meant that the primary beverages on board were beer and wine. Wine is highly acidic and easily stains teeth. Without brushing and rinsing, excessive wine drinking can lead to tooth decay and cavities.

Knowledge about periodontal disease has increase in the last years, and it has been found that its pathogenesis is very complex, and the presence of virulent microorganism is not the only cause of this disorder (Van Dyke and Dave 2005). It is well established that periodontal disease is predominantly a bacterial infection involving the dental biofilm or dental plaque. Several studies have identified the main pathogens of the subgingival microbiome and found that the biofilm that causes this disease is site-specific, a complex polymicrobial community, resistant to the host defense mechanisms and to antimicrobial agents. However, today, it is accepted that the susceptibility to periodontal disease varies greatly between individuals who have the same pathogenic microflora (Armitage and Robertson 2009).

Years ago, researchers believed that all individuals were equally susceptible for periodontal disease (Belting 1957), but with the development of new methodology such as being able to asses depth pocket and clinical attachment loss, several epidemiological studies found differences insusceptibility (?) among different individuals. It was found that some individuals throughout their life presented this disease and many others did not, pointing to the possibility of the existence of other risk factors playing an important role in development of periodontitis (Medina et al., 2019).

Nowadays it is accepted that there are two types of risk factors involved into the ethiopathogeny of the pariodontal disease: modifiable and nonmodifiables.

Modifiable Risk Factors are:

- Microorganisms and Periodontal Disease. The oral bacterial microbiome includes over 700 different phylotypes, with approximately 400 species found in subgingival plaque. The subgingival microflora in periodontitis can harbor hundreds of bacterial species but only a small number has been associated with the progression of disease and considered etiologically important (Paster et al., 2000; Berezow and Darveau 2000).
- Tobacco smoking exerts a substantial destructive effect on the periodontal tissues and increases the rate of periodontal disease progression and modifies the host response to the challenge of bacteria in microbial dental plaque (Shchipkova et al., 2010; Özçaka et al., 2011).
- Diabetes Mellitus (Pucher and Stewart 2004).
- Cardiovascular Disease involvement in periodontal disease is well studied and it includes some of the following possible mechanisms: high concentrations of cholesterol and the

action of oral bacteria in the process of atherosclerosis or the participation of acute-phase proteins that may increase in chronic periodontitis (Izumi et al., 2009; Kamil et al., 2011).

- Drug-Induced Disorders that decrease salivary flow, such as antihypertensives, narcotic analgesics, some tranquilizers and sedatives, antihistamines, and antimetabolites (Cohen 2000; Rees and Levine 1995).
- Stress is associated with poor oral hygiene, increased glucocorticoid secretion that can depress immune function, increased insulin resistance, and potentially increased risk of periodontitis (Marcenes and Sheiham 1992).
- Obesity in younger adults which have different dietary patterns than older study participants. Researches showed significant decrease in raw fruit and nonpotato vegetables, which are sources of vitamin C, decreased calcium intake and increased intake of soft drinks and noncitrus juices (Neiva et al., 2003).
- Socioeconomic Status (SES) - gingival health is better among individuals with higher education and with more secure income. SES is a modifiable factor and it can be examined in multivariate models for the disease (Beck et al., 1990).
- Education and Race - the higher the educational level, the lower the periodontal diseases (Gilbert 2005).

Between Nonmodifiable Risk Factors there are:

- Osteoporosis was significantly associated with severe alveolar crestal bone loss and the prevalence of periodontitis cases in postmenopausal women (Zachariasen 1993).
- Hematological Disorders - patients with chronic leukemia may experience similar but less severe periodontal changes. Chemotherapy or therapy associated with bone marrow transplantation may also adversely affect the gingival health (Waltimo et al., 2005).
- Host Response - The bacteria activate the macrophages to produce cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF). These cytokines activate the fibroblasts that reside in the periodontal tissues to the matrix metalloproteinases (MMPs), which can activate plasmin. Plasmin, in turn, can activate some other types of latent MMPs, while tissue inhibitors of metalloproteinases (TIMPs) can inactivate the active MMPs. MMP-13 also facilitates bone resorption by degrading the collagenous matrix of the bone after the bone is demineralized by osteoclast (Page 1991, Greenstein and Rethman 1996)
- Female Hormonal Alterations that may occur during puberty, the menstrual cycle, pregnancy, or menopause. Changes may also be associated with the use of oral contraceptives. The most pronounced periodontal changes occur during pregnancy, as a significant proportion of pregnant women suffer from pregnancy gingivitis. Women on hormonal replacement therapy (HRT) and oral contraceptives experience increased gingival inflammation (Tiemann et al., 2007).
- Pregnancy. Offenbacher et al. found significantly more periodontal attachment loss among mothers of PLBW infants compared with mothers of normal-term infants (Offenbacher et al., 1996). It has been suggested that periodontal disease may increase the

risk of having preterm low birth weight (PLBW) infants. This outcome is thought to be the effect of biologic mediators of inflammatory processes such as prostaglandins E2 and TNF. The common bacterial product lipopolysaccharide also may have a triggering role in adverse change of the course of pregnancy (Vogt et al., 2010; Bhuyan et al., 2022).

- Age - periodontal disease increase with age. Papapanou et al. demonstrated that the mean annual rate of bone loss among the initially 70-year-old subjects was 0.28 mm compared to 0.07 on the 25-year-old individuals (Papapanou and Wennstrom 1989).
- Gender - periodontal destruction is higher among males compared to the female population and this may be a demographic factor, which may interfere with the effects of other factors and it must be controlled for investigating the disease (Meisel et al., 2008; Al Jehani 2014).
- Genetic Considerations - the composite IL-1 genotype is significantly associated with the severity of adult periodontitis. They also confirmed that both IL-1 genotyping and smoking history provide objective risk factors for periodontal disease in a private practice environment (McDevitt et al., 2000; Emecen-Huja et al., 2019; Kugaji et al., 2020).
- Chronic and aggressive periodontal diseases are complex diseases with multifactorial etiology, that result in the destruction of the supporting structures of teeth. The immune system of the host plays an important role in this process. As environmental risk factors are smoking habits, nutrients and food diet, obesity and involved metabolic syndromes, stress and depression. Concerning genetic risk factors, several studies show the existence of familial aggregation and polymorphisms of diverse genes as well as epigenetic changes have been associated with increased susceptibility to periodontitis (Medina 2019).

Modern treatment of dental loss is dental implant. Periodontal disease could occur after dental implant, as well, in the shape of periimplantitis. It is generated by similar risk factors as common periodontal disease but has its own etiology.

Lack of specific **Maintenance Therapy** is one of them. Supportive therapy has been shown to significantly lower the risk of peri-implant biological complications, and a minimum recall interval of 5–6 months has thus been recommended. Maintenance programs should be tailored to the individual's specific needs and susceptibility to both periodontal and peri-implant diseases. Factors used for risk assessment include the percentage of peri-implant probing, the prevalence of active residual pockets, oral hygiene level, smoking habits and the presence of systemic or genetic conditions. Individuals with high-risk profiles require three to four annual visits, and their attendance is detrimental for prevention and early detection of peri-implantitis (Lang et al., 2015).

Occlusal overload of implant-supported prostheses is a controversial subject, and the exact mechanism in which it causes marginal bone loss is still debatable. Yet several studies have demonstrated that overloading an implant beyond a certain threshold leads to marginal bone loss. Moreover, under similar overload, peri-implantitis-affected sites show significantly higher marginal bone loss compared with those with mucositis (Gotfredsen et al., 2002).

The influence of the material of the implant and **implant's surface topography** on its susceptibility to peri-implantitis is still debatable. The implant's roughness and surface energy

have an impact on initial biofilm formation, but its long-term effect on the inflammatory process and bone stability is still controversial. Few studies demonstrated that rough implants were more susceptible to peri-implantitis (Dank et al., 2019).

The soft tissue condition around an implant may influence its susceptibility to peri-implant disease. Patients with thin periodontal phenotypes are more prone to peri-implant mucosal recessions. The exposure of an implant's rough surface to the oral cavity complicates plaque control and enhances bacterial adhesion, thus leading to a potential increase in its susceptibility to peri-implantitis. A recent clinical study had demonstrated a significant association between thin biotypes and the severity of peri-implantitis (Isler 2019).

Iatrogenic Factors - While the number of implants does not seem to influence the risk for peri-implantitis, their position is critical for long-term success. Implant malpositioning represents a significant risk factor for peri-implantitis. Crestal bone resorption could occur when an implant is placed too close to the natural teeth or even other implants. This could compromise access for plaque control, and thus increase the risk of peri-implant disease. Also, fixtures located outside the bony envelope or those with thin facial bone (< 1 mm) are more prone to mucosal recession, especially in patients with thin biotypes (Passoni et al., 2014; Canullo et al., 2016).

Bio-Corrosion and Presence of Titanium - Particles Despite the availability of zirconia implants, titanium remains the material of choice in implantology. However, the release of titanium particles, and their impact on peri-implant tissues, has recently become subjects of heated debates. Mechanical wear, chemical corrosion and implant surface treatment have been suggested as sources of titanium in the oral environment. The term « tribocorrosion » has been used to describe the combination of wear and corrosion processes. More specifically, corrosion can be observed once mechanical wear has disrupted the protective titanium oxide layer (Delgado-Ruiz and Romanos 2018).

Not all high-risk implants, nor those placed in highly susceptible patients, will develop peri-implantitis. Nevertheless, identifying susceptible implants and patients will help in the tailoring supportive treatment to the patient's need, thus contributing to the primary prevention of the disease. Clinicians should be conscious of the risk periodontal disease present for future biological complications. They should also consider the ramifications of their implant and prosthetic choices, weighing their advantages against their risks (Hashim and Cionca, 2020).

The published articles included included in this research direction have, according to Clarivate, a cumulative impact factor (CIF) of:

This research direction has been materialized by publishing the articles from bellow.

1. Nazemisalman, B.; Mohseni, M.; Darvish, S.; Farsadeghi, M.; **Luchian, I.** Effects of Iron Salts on Demineralization and Discoloration of Primary Incisor Enamel Subjected to Artificial Cariogenic Challenge versus Saline Immersion. *Healthcare* **2023**, *11*, 569. https://doi.org/10.3390/healthcare11040569
2. Budală, D.G.; Balcoș, C.; Armencia, A.; Virvescu, D.I.; Lupu, C.I.; Baciu, E.R.; Vasluianu, R.I.; Tatarciuc, M.; **Luchian, I.** Does the Loss of Teeth Have an Impact on Geriatric Patients' Cognitive Status? *J. Clin. Med.* **2023**, *12*, 2328. https://doi.org/10.3390/jcm12062328
3. Anton, D.-M.; Martu, M.-A.; Maris, M.; Maftei, G.-A.; Sufaru, I.-G.; Tatarciuc, D.; **Luchian, I.;** Ioanid, N.; Martu, S. Study on the Effects of Melatonin on Glycemic Control and Periodontal Parameters in Patients with Type II Diabetes Mellitus and Periodontal Disease. *Medicina* **2021**, *57*, 140. https://doi.org/10.3390/medicina57020140
4. Zaharescu, A.; Mârțu, I.; **Luchian, I.;** Mârțu, M.; Șufaru, I.; Mârțu, C.; Solomon, S-M. 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**, 277. https://doi.org/10.3892/etm.2021.9708
5. Cojocaru, K.-A.**; Luchian, I.;** Goriuc, A.; Antoci, L.-M.; Ciobanu, C.-G.; Popescu, R.; Vlad, C.-E.; Blaj, M.; Foia, L.G. Mitochondrial Dysfunction, Oxidative Stress, and Therapeutic Strategies in Diabetes, Obesity, and Cardiovascular Disease. *Antioxidants* **2023**, *12*, 658. https://doi.org/10.3390/antiox12030658
6. Kappenberg Niţescu, D.C.; Păsărin, L.; Mârţu, S.; Teodorescu, C.; Vasiliu, B.; Mârţu, I.; **Luchian, I.;** Solomon, S.M. Determining Chemotherapy Agents in Saliva through Spectrometry and Chromatography Methods Correlated with Periodontal Status in Oncology Patients. *Appl. Sci.* **2021**, *11*, 5984. https://doi.org/10.3390/app11135984
7. Goriuc, A.; Sandu, D.; Tatarciuc, M.; **Luchian, I.** The Impact of the COVID-19 Pandemic on Dentistry and Dental Education: A Narrative Review. *Int. J. Environ. Res. Public Health* **2022**, *19*, 2537. https://doi.org/10.3390/ijerph19052537
8. Flondor, A.; Martu, M.-A.; Pasarin, L; Maftei, G.-A.; Ciurcanu, O; Toma, V.; Martu, S.; **Luchian, I.** The impact of the Association between Periodontitis and Coronavirus Disease Infection on Oral and Systemic Complications. Review. *Rom. J. Oral Rehab.* **2022,** 14, 4.
9. Martu, M.-A.; Ciurcanu, O; Maftei, G.-E.; Pasarin, L; Popa, C; Sandu, D; Butnaru, O; **Luchian, I.** Hematological Pathology between Diagnosis and Treatment in the Context of Oral Manifestations. Management of the Patient of Leukemia in the Dental Practice. Review. *Rom. J. Oral Rehab.* **2022**, 14, 4.
10. Maftei G.-A.; Ciurcanu, O; Luca, O; Pasarin, L; Popa, C; Mares, M; Martu M.-A.; **Luchian, I.** Are Perodontal Tissues and Periodontal Health Impacted by Food Supplements (protein and aminoacids, natural herbal products, minerals)? – Review. *Rom. J. Med. Dent. Educ.* **2022**, 11, 5.
11. Martu, M.A.; **Luchian, I;** Ciurcanu, O; Luca, O; Mares, M; Pasarin, L; Popa, C; Martu, I. Influence of Fish Oil/Omega-3 Fatty Acids Food Supplements on Periodontal Tissues and Health. A Narrative Review. . *Rom. J. Med. Dent. Educ.* **2022**, 11, 5.

1.2. Nutrition influence on periodontal status

1.2.1. Introduction

Physical and psychological growth and development is a dynamic process that greatly depends on proper nutrition (Mehran et al., 2009). The use of food supplements and so-called superfoods to increase fitness and regeneration or just to improve health and well-being is very popular these days, particularly in people living the fitness lifestyle. Some of the effects attributed to these supplements and superfoods involve tissues and processes that may also play a role in periodontal healing and regeneration. However, some the current evidence is of a very low quality, and more validated scientific data are required before their possible use in prevention or treatment of periodontal diseases can be made. Periodontitis is a dysbiotic inflammatory disease and the result of a host immunoinflammatory response to periodontopathic bacteria.

⇒ **Protein and Aminoacid Supplements**

The most frequently used supplements in the fitness lifestyle are protein and amino acid supplements used to support muscle growth and muscle regeneration.

Possible beneficial effects of protein and amino acid supplements have been extensively studied in orthopedic and sports medicine, and also in geriatric medicine, since proteins constitute an important structural and functional component of skeletal tissues. Some of these so-called muscle supplements might also have effects on periodontal tissues as well as on periodontal wound healing. Alteration in protein turnover following tissue damage due to injury or extensive exercise is crucial to tissue repair. Increasing knowledge has indicated the need for increased protein intake during tissue repair based on its important roles supporting wound healing, maintaining tissue integrity, and promoting convalescence (Axel et al., 2000; Martu et al, 2020).

An insufficient protein intake has been shown to delay wound healing and to reduce the integrity of the repaired tissue (Brosnan 2006, Thomson and Buckley 2011, Tui 1945). Thus, milk can be considered as a natural biological liquid esculent providing nutrition at a time of rapid body and particularly muscular-skeletal growth. Total protein intake and animal protein intake have been associated with higher bone mineral density and less bone mineral density loss over time. The positive effects of dietary protein on bone mineral density may be due to increased levels of insulin-like growth factor 1 and suppression of parathyroid hormone.

Further investigation of these whey protein fractions revealed that lactoferrin was a constituent in many of these fractions (Cornish et al., 2003). However, local concentrations can increase during inflammation (Concavo et al., 1999).

Studies of lactoferrin on human, rat, and mouse cell cultures of the osteoblast and osteoclast lineage and of bone marrow cultures showed that lactoferrin promotes osteoblast growth, inhibits osteoclastogenesis, and reduces osteoblast apoptosis. Interestingly, besides its effects on bone metabolism, there are also reports of antimicrobial effects of lactoferrin, attributed to its action as an iron chelator, as well as of an immunomodulatory function. Lactoferrin has been shown to decrease the secretion of interleukin- 1β and tumor necrosis factor

alpha and to stabilize mast cells (Zimecki et al., 1998; Baveye et al., 1999; Weinberg 2001; Kimber et al., 2002; He et al., 2003).

A significant decrease in tumor necrosis factor alpha serum levelsafter administration of a high-caloric protein-rich oral supplement was also reported in a prospective randomized, double-blind, placebo-controlled study in patients with chronic heart failure and cachexia (Da Silva et al., 2017).

This was also confirmed by a clinical trial in sarcopenic elderly patients where supplementation with whey protein, amino acids, and vitamin D increased serum insulin-like growth factor 1 concentrations and lowered C-reactive protein (Rondaneli et al, 2016). Furthermore, protein deficiency may also predispose patients to higher rates of infectious complications (Tkatch et al., 2018). Protein hydrolysates have the potential to promote different types of tissue repair and might be useful in situations where excess protein is needed, such as tissue repair, regeneration, or wound healing. However, further well-controlled human trials are required to confirm these findings and assess the clinical relevance in periodontal therapy.

Lipopolysaccharide of gram-negative bacteria, like most of the main periodontal pathogens, is known to induce the release of proinflammatory cytokines such as interleukin-1β, interleukin-6, interleukin, tumor necrosis factor-alpha, and nitric oxide in macrophages. Furthermore, recent studies have also indicated that branched-chain amino acids may play a role in the development of insulin resistance and might be associated with incident cardiovascular disease (Harris et al., 2005; Bloomgarden 2018; Tobias et al., 2018).

Glutamine has also been shown to simulate collagen synthesis through the conversion process to proline and provides 75% of the intracellular free proline in fibroblasts. Amman et al. (Ammann et al., 2002) investigated the effect of essential amino acid supplements in adult osteoporotic rats. Unfortunately, the number of studies investigating the effect of protein and amino acid supplements on periodontal disease or therapy is very limited.

Aral et al. (2017) investigated the effect of bodybuilding and protein supplements on periodontal tissues, comparing bodybuilders with gingivitis with nonexercising males with and without gingivitis. They assessed clinical periodontal parameters and analyzed saliva and gingival crevicular fluid samples for interleukin-1β, apoptosis-associated speck-like protein containing C-terminal caspase-recruitment domain and caspase 1.

The authors indicated that bodybuilding and supplement usage may decrease gingival inflammation by downregulating caspase 1, interleukin-1β, and apoptosis-associated speck-like protein containing C-terminal caspase-recruitment domain.

Lee et al. (2012) investigated the effects of a commercially available nutritional supplement drink on periodontal health or healing and tooth mobility after periodontal flap surgery.

Patients with a generalized moderate chronic periodontitis were, directly after periodontal flap surgery, randomly allocated to either the intervention or the control group.

After 8 weeks, tooth mobility returned to baseline levels again in both groups.

The authors concluded that the use of nutritional supplementation may improve early periodontal wound healing after periodontal surgery.

⇒ **Glucosamine and Chondroitin Sulfate**

Glucosamine is a naturally occurring amino monosaccharide that is present in the connective tissue and cartilage tissues as a component of glycosaminoglycans and is involved in maintaining strength, flexibility, and elasticity of these tissues. Numerous studies have shown the significant symptom-modifying effect of glucosamine in osteoarthritis and its beneficial effects on joint health (McAlindon et al., 2000; Reginster et al., 2001; Nagaoka et al., 2012,). There are also an increasing number of studies investigating the effects of glucosamine in combination with chondroitin sulfate in osteoarthritis therapy. Supplementation with glucosamine has been shown to reduce inflammatory responses of joint cartilage by inhibiting the activation of nuclear factor kappa-light- chain- enhancer of activated B cells, which lies upstream of inflammatory processes or mediators such as interleukin-beta, interleukin-8, tumor necrosis factor alpha, and C-reactive protein. Binding of proinflammatory cytokines to their respective receptors amplifies immune response by increasing proliferation of T cells, promoting leukocyte infiltration and facilitating cell-cell signaling (Mosmann ans Sad 1996; Largo et al., 2003; Xu et al., 2008; Bak et al., 2014). Cigarette smoking causes lung inflammation that is mainly regulated by redox-sensitivepathways.

It was recommended that glucosamine hydrochloride could be used as a pharmaceutical supplement to alleviate oxidative stress (Jamialahmadi et al., 2014).

Interleukin-8 plays an essential role in directing the sequential process of neutrophil rolling, adhesion, and transmigration into inflamed microvasculature.

Furthermore, proinflammatory mediators, such as interleukin-1β, interleukin-6, and tumor necrosis factor alpha increase the expression of adhesion molecules on endothelial cells and neutrophils (Mosmann ans Sad 1996; Ardite et al., 1998; Largo et al., 2003; Xu et al., 2008; Bak et al., 2014). However, glucosamine also seems to exhibit interesting effects on bone and collagen metabolism. These results indicate that glucosamine increases bone mineral density, induces osteoblastic differentiation, especially at middle and late stages, and also suppresses osteoclastic cell differentiation, thereby increasing bone matrix deposition, decreasing bone resorption, and promoting bone formation (Shimizu et al., 2011).

Furthermore, risk factors for diabetes development are elevated triglycerides, blood pressure, body mass index, and family history of diabetes.

The findings of this study indicate that there is no effect of glucosamine sulfate on mean hemoglobin A1c level or on obtaining a high hemoglobin A1c level or new-onset Diabetes mellitus over 6.5 years, especially in participants with a normal hemoglobin A1c level at baseline.

The results of an in vitro study using inferior nerve preparation in a rat mandible suggest that d-glucosamine hydrochloride has a pain relief effect on patients with dental pain (Kaida et al., 2014).

⇒ **Natural herbal products and seeds "functional food or super food" (**quinoa, spirulina),

In recent years, the increasing number of people suffering from cardiovascular diseases, obesity, diabetes mellitus, neurologic diseases, dementia, cancer, and other related diseases has shifted the focus from disease treatment to healthy lifestyle changes.

Epidemiologic studies have shown that physical inactivity and unhealthy diet containing high amounts of refined carbohydrates combined with saturated fatty acids but lacking fiber, minerals, and antioxidant micronutrients may be an important risk factor in the development of pathologic conditions.

Whereas pre-Neolithic oral bacterial ecosystems were more diverse and dominated by the nonpathogenic family of Ruminococcaceae, modern oral ecosystems are less diverse with an abundance of periodontopathogens, such as P. gingivalis, Tannarella, and Treponema, and cariogenic species, such as Streptococcus mutans (Alder et al., 2013). In 2009, Baumgartner et al. illustrated that diet may have a significant impact on periodontal inflammatory status.

The Swiss study on 10 adults who were placed in a "Stone Age" environment and on diet rich in fibers, fish oils, and micronutrients showed significant reduction in bleeding on probing and pocket depth compared with baseline, even in the absence of oral hygiene (Baumgartner et al., 2009; Ilc et al., 2012). An evidence-based review, based on 31 human studies that explored the relationship between food supplements and periodontitis, showed substantial evidence of beneficial outcomes for treatment of periodontal diseases from nutritional intervention.

It also suggested guidelines for micronutrient supplement intake (mainly vitamins C and D) that may improve results in the treatment of periodontitis, especially in cases of refractory disease (Van der Velden et al., 2010). Furthermore, it has been shown that the use of nutritional agents as adjuvants to nonsurgical periodontal therapy significantly reduced the periodontal disease severity, improved treatment prognosis in the short term (2-6 months), and reduced susceptibility toward periodontal disease (Sousa et al., 2020).

In the past few years, emerging evidence from the studies have increased the awareness of the industry and consumers related to the possible nutritional and health attributes of certain natural herbal products. Apart from that, it is a source of many vitamins (thiamin, riboflavin, niacin, and folic and ascorbic acids) and minerals (calcium, phosphorus, potassium, and magnesium), as well as compounds with antioxidant properties (Ali et al., 2012; Ixtaina et al., 2008).

⇒ **Chia** seeds extract has been tested in a recent in vitro study and demonstrated excellent antimicrobial efficacy against three periodontal pathogens: P. gingivalis, Aggregatibacter actinomycetemcomitans, and F. nucleatum. Its inhibitory potential was similar to 0.2% chlorhexidine, which was used as positive control (Divyapriya et al., 2016).

As there are no clinical trials related to the preventive or therapeutic properties of chia seeds on the diseases that affect oral mucosa and periodontium, we can only suggest that the systemic anti-inflammatory potential of chia seeds shown in some studies may play a role in the prevention and treatment of periodontal diseases.

It can also be stipulated that their antioxidative potential may have an effect on oxidative stress that orchestrates proinflammatory cascades that underpin tissue destruction in periodontitis

and other inflammatory conditions associated with periodontitis, such as type 2 diabetes, cardiovascular disease, and obesity and related metabolic dysregulation.

The seeds' mineral content may also improve the quality of bone and prevent osteoporosis and its effect on the periodontal status of the patients.

Quinoa seeds, leaves, and sprouts are used as human and animal food owing to their nutritional values.

Quinoa has been described as "one of the grains of the 21st century," and its production, preservation, and consumption were promoted by the Food and Agriculture Organization of the United Nations in 2013. Quinoa is superior to many grains, such as rice, rye, barley, and oat, in relation to protein and lipid content. It contains 13.1%- 16.7% of high-quality proteins with well-balanced essential amino acid content that satisfies the amino acid requirements for adults suggested by the Food and Agriculture Organization of the United Nations/World Health Organization/United Nations University.

It contains a significant amount of essential amino acids, such as lysine, methionine, and threonine, that is higher than in essential cereals, such as wheat and maize. The content of micronutrients, such as vitamins and minerals, is also of great importance, as the seed is rich in pyridoxine (vitamin B6), folic acid, ascorbic acid (vitamin C), and vitamin E. Mineral content, such as calcium, iron, and magnesium, is considerably higher than in other commonly used grains, such as wheat and corn.

These components are known to exhibit a wide range of health benefits, such as antifungal, antiviral, antibacterial, and cancer-suppressing effects.

They also exhibit hypoglycemic, antithrombotic, diuretic, anti-inflammatory, anabolic, antidiabetic, anti-osteoporotic, and anti-obesity properties (Vilcacundo et al., 2017).

The evidence of some of these benefits is demonstrated in limited numbers of animal and human studies.

Vitamins: thiamine, riboflavin, niacin, folic and ascorbic acids Minerals: calcium, phosphorus, potassium, and magnesium Antioxidants: polyphenols, chlorogenic acid, caffeic acid, quercetin, kaempferol Fatty acids: omega-3 and omega-6 fatty acids (Ali et al., 2012; Ixtaina et al., 2008) Improvement of lipid profile; reduction of risk of diabetes and cardiovascular diseases (Vuksan et al., 2007).

⇒ **Quinoa** (Cehnopodium quinoa Wild.) Vitamins: pyridoxine (vitamin B6), folic acid, ascorbi acid, and vitamin E. The antibacterial activity of quinoa against oral bacteria has rarely been reported. A recent in vitro study showed that alkali- transformed saponins derived from quinoa husk were efficient against three halitosis-related bacteria: *P. gingivalis, Clostridium perfringens*, and *F. nucleatum*. The saponins altered membrane potential and morphology, as well as interfered with its permeability, causing leakage of nucleic acids and proteins. The results of the study indicated that saponins derived from quinoa husk may have an important role in a new drug delivery system against oral halitosis caused by oral microorganisms (Pasko et al., 2010).

⇒ **Spirulina**. Arthrospira platensis is a microscopic single-cell alga that inhabits fresh and marine

waters.

Spirulina contains up to 70% of proteins; it is also rich source of vitamins (B-complex, β-carotenes, and vitamin K) and minerals (iron, magnesia, zinc, copper, selenium, and chromium). It can be easily cultivated, harvested, and processed into a variety of final products, such as powder, tablets, flakes, and other edible profiles (Ravi et al., 2010). Owing to the high content of carotenoids and the protein-bound pigment C- phycocyanin this blue-green algae has been shown to have antioxidant and immunomodulatory properties in in vitro and in vivo studies. These substances may act as scavengers of reactive oxygen species mainly generated by host defense cells during an inflammatory reaction and increased oxidative stress (Ravi et al., 2010).

Oxidative stress was first described by Sies in 1985, and some years later it was revealed that it underpins the pathogenesis of numerous of inflammatory diseases, such as periodontitis, diabetes, cardiovascular disease, and obesity/metabolic dysregulation (Sies and Cadenas 1985).

The antioxidative potential of spirulina has been demonstrated in several human studies conducted on geriatric patients and onhealthy individuals after exercise.

Food supplemented by spirulina for 16 weeks showed significantly increased levels of antioxidant status in plasma of geriatric patients (Park et al., 2008).

In addition to its unproven role as an antioxidant and immunomodulator, spirulina has been reported to improve blood lipid profile, which may be of importance in prevention of diabetes and cardiovascular diseases (Ravi et al., 2010).

Recently, Spirulina (Arthrospira) maxima was tested on rats as a potential agent in treatment of periodontitis. Gingival tissue of rats treated with S. maxima showed reduced concentrations of proinflammatory cytokines, such as tumor necrosis factor alpha, interleukin-1β, interleukin-6, and inflammatory transcription factor nuclear factor kappa-light- chain- enhancer of activated B cells. Activity of myeloperoxidase and expression of matrix metalloproteinases were also decreased in periodontal tissue of test rats.

In addition, treatment with S. maxima increased concentration of anti- inflammatory cytokine interleukin-4 and the osteoprotegerin/ RANKL expression ratio. S. maxima-treated groups showed reduced numbers of osteoclasts and less bone loss, as well as increased production of osteoblasts and osteogenesis-related factors (Kang et al., 2021).

Scarce evidence so far exists on the effect of spirulina on periodontal health and disease. One randomized controlled clinical study tested the benefits of local application of spirulina-based

Gel as adjunct to nonsurgical treatment (scaling and root planing) of chronic periodontitis.

The results showed significant improvement of clinical parameters, such as probing depth reduction and clinical attachment gain, in the experimental group when compared with the control group (scaling and root planing alone). During the course of treatment, spirulina gel did not cause any side or adverse effects (Mahendra et al., 2013).

As this study is one of the first to use spirulina as a local adjunct agent in the treatment of periodontitis, further studies, including more relevant clinical and biochemical parameters, are necessary to confirm the findings and explore underlying mechanisms.

⇒ **Turmeric** is a dietary spice whose active ingredient, curcumin, is isolated from the rhizomes

of Curcuma longa, a plant that belongs to the ginger family. Turmeric is yellow in color and is most used in Asian and Indian cuisine.

Curcumin has been approved by the US Food and Drug Administration to be a safe food supplement, and a daily intake of curcumin at a dose of 0.1-3 mg/kg body weight has been considered as an acceptable dose by the Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives, 1996. (Ghosh et al., 2015).

Curcumin (mainly its analogues) has shown chemopreventive and chemotherapeutic properties in different cancer studies.

It has been shown in vivo (paw edema model) to have analgesic and anti- inflammatory activity through suppression of gene expression and inhibition of secretion of proinflammatory substances, such as tumor necrosis factor alpha, monocyte chemoattractant protein-1, interleukin-10, and interleukin-6.

It has been delivered in the form of nanoparticles, tablets, capsules, powder, or solution in doses from 0.18 to 8 g/d. (Prasad et al., 2014). In clinical trials, patients with periodontal diseases usually use curcumin as adjuvant therapy following subgingival instrumentation.

All trials were conducted in India, with the main objective to compare the efficacy of turmeric and chlorhexidine, formulated either as a mouthwash or gel, in the prevention and treatment of gingivitis (Stoyell et al., 2016). Three studies on prevention showed that, as an adjunct to mechanical plaque control, turmeric-based mouthwash significantly reduced plaque accumulation and gingival inflammation after the experimental period (14-21 days).

However, when compared with chlorhexidine, it was less efficient (Waghmare et al., 2011). Two studies tested the therapeutical efficacy of turmeric and chlorhexidine as an adjunct to mechanical treatment of gingivitis and compared them with mechanical treatment alone.

It has been widely used in photodynamic therapy of cancers owing to its low cost and high efficacy. De Paula Zago et al., have shown that curcumin can significantly inhibit the growth of oral pathogens while used as a photosensitizer.

In a clinical study, Sreedhar et al. (2020) used curcumin gel as a photosensitizer in photodynamic therapy following subgingival instrumentation with ultrasonic scaling in 15 patients with deep periodontal pockets.

Curcumin showed enhanced antimicrobial properties against P. gingivalis, A. actinomycetemcomitans, and Prevotella intermedia.

These results were improved when the multiple applications of photodynamic therapy were performed. The curcumin binds to the cell wall of periodontal pathogens and when irradiated with light of specific wavelength produces reactive oxygen species, which can destroy the pathogens in the immediate vicinity (Sreedhar et al., 2015). This and other preliminary in vivo studies provide initial evidence that curcumin may offer periodontists a complementary approach to the conventional periodontal therapy through either systemic or local application (Zbou et al., 2013).

⇒ **Açai-berry**, the fruit of the Amazonian palm, Euterpe oleracea Martius, has been extensively

studied not only for its nutritional properties but also its anti- inflammatory, antioxidant, and bioactivity properties. Açai pulp fraction contains a remarkable number of phytochemicals and mono- and polyunsaturated fatty acids (Jensen et al., 2008). Phytochemical analyses indicate that açai extract is rich in anthocyanins and possesses a high number of polyphenols, especially flavonoids, that exhibit promising therapeutic potential.

Earlier in vitro studies demonstrated that acai extract may exhibit potent anti-inflammatory,
neuroprotective, and anticarcinogenic properties (Jensen et al., 2008). These findings may be of importance for further testing and development of novel therapeutic agents with potential to reduce inflammatory bone loss that occurs as a result of periodontitis.

⇒ **Minerals**

Minerals belong to the group of minor/micronutrients that are present in food in small amounts, measured by microgram quantities. Minerals act as catalysts in a variety of enzyme systems, either as ionic enzymatic cofactors or metalloenzymes. Regular daily intake of food rich in minerals is usually sufficient to maintain health; however, in some cases, pharmacological supplements are used to maintain satisfactory levels or treat deficiencies (Robert 2000). Sodium is the cation and main major mineral in extracellular fluid. It plays a key role in cellular membrane potential and nerve conduction, and together with calcium, potassium, and magnesium has an important influence on cardiac output and peripheral vascular resistance, the main determinants of blood pressure level (Karppanen et al., 2005). Sodium is mainly consumed as sodium chloride, "dietary salt," but may be found in food additives, too.

Potassium is the key cation in intracellular fluid with a similar role to sodium. Potassium is known to have a protective effect on the cardiovascular system, and its anti-atherosclerotic
properties have attracted attention in the recent years. Other health benefits of potassium may be related to diabetic patients and improvement of their glucose tolerance (Robert 2000).

Calcium is the main component of hydroxyapatite, a mineral that is present in our skeletal system and teeth. It is important for normal bone turnover, nerve conduction, and blood coagulation (Karppanen et al., 2005). Metabolism of calcium is regulated by parathyroid hormone and calcitonin, and its active resorption through intestinal wall is highly dependent on vitamin D.

Numerous clinical studies have emphasized the importance of calcium intake in bone mineral density maintenance and tooth retention, especially in the elderly population.

Vitamin D deficiency is common in the world, with an estimate that more than 1 billion people suffer from its insufficiency or deficiency (Crockett et al., 2018). The beneficial effect of supplementation with vitamin D and calcium has been well documented and recognized in the treatment of rickets, osteomalacia, and osteoporosis. In recent times, vitamin D and calcium have also been considered as candidates to modulate periodontal disease, as some studies have found that their intake may reduce alveolar bone loss, gingival inflammation, and attachment loss. Caution should be considered with patients reporting a risk of bowel cancer (Crockett et al., 2018).

The Third National Health and Nutrition Examination Survey large cohort of up to 12 000 subjects suggested that low dietary intake of calcium results in more severe periodontal disease and progressive attachment loss in a dose- dependentmanner (Miley et al., 2009).

Another study that used data from the Third National Health and Nutrition Examination Survey reported an inverse association between the prevalence of periodontal disease and the intake of dairy products, a common dietary source of calcium and vitamin D (Al-Zahrani 2006).

A recent cross-sectional study on 51 subjects on periodontal maintenance therapy resulted in a trend toward better clinical (gingival inflammation, probing depth, and attachment loss and furcation involvement) and radiological parameters (cemento-enamel junction to alveolar crest distance) of periodontal disease in patients who were voluntarily taking calcium (at least 1000 mg/day) and vitamin D (at least 400 IU/day) supplements for more than 18 months (average of 10.6 years) prior to commencement of the study (Miley et al., 2009).

Although some studies implied benefits of daily supplementation with vitamin D and calcium, use of these microelements in healthy patients with periodontal disease requires further evidence. Recommended daily intake of calcium for adults ranges from 1000 to 1300 mg/day.

Magnesium is second most prominent intracellular cation and is present in all tissues, with majority (two-thirds) stored in bones. Imbalances in magnesium metabolism may be associated with different pathologic conditions such as cardiovascular diseases, diabetes, pre-eclampsia, eclampsia, and sickle cell disease (Van der Velden et al., 2010). Low magnesium intake has been linked to periodontitis (Staudte et al., 2012).

In a cross-sectional epidemiologic study involving 4290 subjects from 20 to 80 years of age, periodontal health was determined and correlated to concentrations of serum magnesium and calcium. In a matched study, the periodontal status of 60 subjects from the same population using magnesium drugs was compared with 120 nonusers. Subjects taking magnesium showed less attachment loss ($P < 0.01$) and a higher number of remaining teeth than did their counterparts. The findings of the study indicate that magnesium supplementation may improve periodontal status and improve tooth retention (Meisel et al., 2005).

⇒ **Fish Oil/Omega-3 Fatty Acids on periodontal tissues and health**

Fish oil, and particularly the enclosed omega-3 polyunsaturated fatty acids, is assumed to be beneficial for human fitness and well-being. Their wholesome effects are claimed to promote or participate in heart and vascular health, brain or neurological development and function, mental health and function, vision, immune system balance, body weight control, joint function, and bone and muscle mass and strength (Dawson et al, 2000; Lau et al., 2005; Harris et al., 2008; Buckley and Howe 2009; Galland et al., 2010; Hamazaki et al., 2013; Kajarabille et al., 2013; Serhan 2014; Byelashov et al., 2015).

Therefore, fish-oil supplements or other supplements rich in omega-3 polyunsaturated fatty acids are today one of the most common and widely used dietary supplements in the health and fitness sector. There are two classes of essential fatty acids, omega-3 and omega-6.

Omega-3 and omega-6 fatty acids are polyunsaturated fatty acids containing more than one cis double bond that is located between the third and the fourth carbon atoms from the

methyl end in omega-3 fatty acids and between the sixth and seventh carbon atoms in omega-6 fatty acids. The main natural dietary sources for alpha-linolenic acid are plant-based sources such as green leafy vegetables, flaxseed, chia seeds, canola, soybeans, walnuts, and pecans, but also chicken and beef.

However, the claimed health benefits associated with fish oil have been attributed. Furthermore, lifestyle factors, like alcohol consumption, have also been shown to reduce docosapentaenoic acid levels in the plasma and the liver (Pawlosky and Salem, 1995).

Therefore, supplement capsules could be a good source of long-chain omega-3 olyunsaturated fatty acids for people who cannot or are not keen to eat a sufficient amount of fish or seafood (Miller et al., 2012; Rahmawaty et al., 2013; Linus 2014). Current intake recommendations regarding omega- 3 polyunsaturated fatty acids, and particularly eicosapentaenoic acid and docosahexaenoic acid, vary between different organizations and for different indications.

The European Food Safety Authority recommends an adequate intake of 250 mg/day for eicosapentaenoic acid plus docosahexaenoic acid.

The World Health Organization recommends an acceptable macronutrient distribution range for eicosapentaenoic acid plus docosahexaenoic acid of 250 mg/day to 2 g/ day (the upper limit applying to the secondary prevention of coronary heart disease).

The International Society for the Study of Fatty Acids and Lipids recommends for healthy adults a minimum of 500 mg/day of eicosapentaenoic acid plus docosahexaenoic acid for cardiovascular health.

The American Heart Association recommendation for people without documented coronary heart disease is to eat fish at least twice weekly, providing approximately 500 mg of eicosapentaenoic acid plus docosahexaenoic acid.

People with documented coronary heart disease are advised to consume approximately 1 g/day of eicosapentaenoic acid plus docosahexaenoic acid, preferably from oily fish or to consider supplements in consultation with a physician. Omega-3 and omega-6 polyunsaturated fatty acids are important structural components of the phospholipids of all cell membranes.

After stimulation by hormones, cytokines, or other factors, long-chain polyunsaturated fatty acids are released from cell membranes and become substrates for eicosanoid production.

Eicosanoids generated from long-chain omega-6 polyunsaturated fatty acids, like arachidonic acid, are considered mainly proinflammatory.

By contrast, eicosanoids generated from long-chain omega-3polyunsaturated fatty acids are less potent inducers of inflammation, blood vessel constriction, and coagulation and are, therefore, considered as antiinflammatory.

Both eicosanoids show a high inflammatory potential, which increases production of interleukin-6 and enhances vascular permeability and vasodilatation. Leukotriene B4 recruits neutrophils to areas of tissue damage, increases the production of interleukin-1 and tumor necrosis factor alpha, and induces the release of reactive oxygen species from leukocytes (Dawson et al., 2000; Raffaelli et al., 2008).

However, it would be an oversimplification to describe all arachidonic acid–derived eicosanoids as proinflammatory. Although arachidonic acid–derived prostaglandins induce inflammation they also inhibit proinflammatory leukotrienes and cytokines and induce anti-inflammatory lipoxins.

Their expression results in decreased vascular permeability and vasodilatation as well as reduced immune cell recruitment (Kunsella et al., 1990; Caughey et al., 1996; Dawson et al., 2000; Serhan et al., 2008).

Dietary supplements rich in omega-3 polyunsaturated fatty acids have been shown to reduce the concentration of 2- series prostaglandins and increase the synthesis of 3-series prostaglandins, which are suggested to be less inflammatory.

Their study revealed that prostaglandin 3, unlike prostaglandin E2, is not mitogenic to NIH

Additionally, more recent studies revealed that long-chain omega-3 polyunsaturated fatty acids also serve as substrates for enzymatic conversion to a novel series of bioactive lipid mediators with antiinflammatory, inflammation-resolving, and protective capabilities (Serhan et al., 2002; Chiang et al., 2020).

It is now widely recognized that resolution of inflammatory responses is an active and not, as previously considered, a passive process. Increasing evidence shows that the resolution phase is a biosynthetically active process that is governed by a superfamily of specialized pro-resolving mediators. Resolvin E1 was the first discovered specialized pro-resolving mediator derived from eicosapentaenoic acid, identified during the resolution phase of acute inflammation.

The E-series resolvins display potent anti- inflammatory and immunoregulatory properties, control acute and chronic inflammation, neurologic disorders, and cancer, as well as stimulate tissue repair. Protectin D1/neuroprotectin D1 is biosynthesized from docosahexaenoic acid and has demonstrated neuroprotective actions in the brain, retina, and central nervous system. The aspirin-triggered epimer has been shown to control polymorphonuclear neutrophils, enhance macrophage functions, and attenuate experimental stroke (Schwab and Serhan 2006; Serhan 2014; Chiang et al., 2020).

Both resolvins E1 and D1 and protectin D1 have demonstrated activity as regulators, inhibiting the migration of neutrophils from capillaries and also limiting neutrophil infiltration into inflamed tissue, thus supporting the resolution of inflammatory processes. Furthermore, they seem to inhibit the production of tumor necrosis factor alpha and interleukin-1β, and there are reports about an additive effect of protectin D1 when acting in concert with resolvin E1 (Bannenberg et al., 2005; Arita et al., 2005; Duffield et al., 2006).

Resolvins and protectins are also generated in their respective epimeric forms when aspirin (acetylsalicylic acid) is given in mammalian systems. In the presence of aspirin, eicosapentaenoic acid and docosahexaenoic acid undergo transcellular metabolism in human cells to release various anti-inflammatory, pro- resolution, lipid mediators. Aspirin modifies the activity and specificity of cyclooxygenase-2 and seems to be critical to the enhanced activity of the stereoisomers (18(R)- vs 18(S)-resolvins) (Vardar et al., 2005; Kesavalu et al., 2006; Kesavalu et al., 2007; Filion et al., 2010).

The number of studies investigating possible health benefits due to the intake of fish oil or omega-3 polyunsaturated fatty acid supplements has increased significantly during the last decade. Possible beneficial effects of an adjunct use of fish oil or omega-3 polyunsaturated fatty acids have been investigated for visual and neurologic development, gestation and pregnancy,(Szajewska et al., 2006; Horvath et al., 2007; Kromhout 2012) cardiovascular disease or coronary heart disease and atherosclerosis (Kalmijn et al., 2003; Mozaffarian and Wu 2011).

Alzheimer disease or dementia, (Farmer et al., 2001; Morria et al., 2003) diabetes, (Montori et al., 2000; Lee et al., 2012; Jeppesen et al., 2013) rheumatoid arthritis, (Cabre et al., 2012) Crohn disease or ulcerative colitis, (Reisman et al., 2006; Swan and Allen 2013) asthma, (Thien et al., 2002; Liu et al., 2012) immunoglobulin A nephropathy, (Hibbeln 1998) neuropsychiatric disorders like depression, bipolar disorder, or schizophrenia,)Hoen et al., 2013, Locke and Stoll 2001, Prior and Galduróz 2012, Siena et al., 2018) and also cancer (Hajishengallis 2015; Zanoaga et al., 2017; Vernieri et al., 2018).

Based on the improved understanding of function and beneficial effects of fish oils or omega-3 polyunsaturated fatty acid supplements in the numerous systemic diseases mentioned earlier herein, attention has been drawn to the investigation of possible beneficial effects on oral tissues or diseases. The antiinflammatory effects, an adjunct in prevention and treatment of periodontal disease. Periodontitis is a dysbiotic inflammatory disease and the result of a host immuno- inflammatory response to periodontopathic bacteria. The destruction of periodontal tissues is characterized by an inflammatory neutrophil-mediated tissue injury followed by chronic infiltration of monocytes and the establishment of an acquired immune lesion.

Although initiated by periodontopathic bacteria, investigations of the pathogenetic mechanisms associated with periodontal diseases have shown that the largest amount of periodontal tissue damage is not caused by bacteria directly but by the host response to infection. Important mediators in periodontal tissue destruction are prostaglandins and leukotrienes, produced from the metabolism of arachidonic acid. Attention has been focused on the role of prostaglandin E2, which, in addition to its proinflammatory action by stimulating various proinflammatory cytokines, also participates in the destruction of alveolar bone and periodontal connective tissue by activating osteoclasts and increasing the synthesis of matrix metalloproteinase-1 (Page 1991; Alam et al., 1991; Offenbacher 1996; Reddy et al., 2003; Van Dyke et al., 2003).

Therefore, it is not surprising that the number of studies investigating the effect of omega-3 polyunsaturated fatty acids on prevention and treatment of periodontal diseases has increased significantly over the last decade.

Alam et al., (1991) showed in an early study in rats that animals fed with a diet rich in omega-3 polyunsaturated fatty acids exhibit reduced levels of arachidonic acid, prostaglandin 2, and leukotriene B4 in gingival tissue. Campan et al investigated the effect of omega-3 polyunsaturated fatty acids in the treatment of human experimental gingivitis. The levels of arachidonic acid, prostaglandin E2, and leukotriene B4 were decreased in the experimental fish oil group and increased in the olive oil control group, but with no significant difference. Clinically, there was a significant reduction of the gingival index in the test group but no

significant difference between the groups. Eberhard et al. (2014) investigated the effect of a topical application of omega-3 or omega- 6 polyunsaturated fatty acids in a human experimental gingivitis model.

The subjects were randomly assigned to two groups using a mouthrinse enriched in omega-3 polyunsaturated fatty acids or an omega-6 soya oil solution. However, bleeding on probing, gingival crevicular fluid, and leukotriene B4 levels were significantly increased in all groups with no differences between the control and A study by Bendyk et al investigated the effect of omega-3 polyunsaturated fatty acids on experimental periodontitis in mice. After 14 days, the mice were inoculated orally with Porphyromonas gingivalis alone or a mixture of P. gingivalis and Fusobacterium nucleatum suspended in carboxymethyl cellulose, with carboxymethyl cellulose alone or remained untreated. At the end of the observation period the mice were killed, soft-tissue biopsies of the oral cavity used for measurement of the polyunsaturated fatty acid concentrations, and the maxilla removed, stained, and digitally imaged to assess the bone loss around the upper molars.

After 57 days, there were marked differences in the polyunsaturated fatty acid contents of the intra-oral soft tissues of mice fed with the tuna oil–enriched diet compared with those fed with Sunola oil. The oral tissues of the tuna oil fed mice showed 10-fold increased eicosapentaenoic acid levels and twofold increased docosahexaenoic acid levels, whereas the levels of omega-6 polyunsaturated fatty acids were halved.

Furthermore, tuna oil–fed mice inoculated with P. gingivalis alone or the combination of P. gingivalis and F. nucleatum showed 72% and 54% less alveolar bone loss, respectively, compared with the treatment control group.

Similar observations were reported in a study by Kesavalu et al, (2007) where rats were fed with fish oil or corn oil and infected with P. gingivalis.

The corn oil diet contained 60% omega-6 linoleic acid and the fish oil diet 24.6% omega-3 polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid). Experimental periodontitis in this study was induced by repeated injections of Escherichia coli lipopolysaccharide. Both omega-3 polyunsaturated fatty acid groups showed no reduction in lipopolysaccharide-induced alveolar bone loss compared with the lipopolysaccharide control group, significantly higher interleukin-1β and osteocalcin levels, and no effect on serum C-reactive protein level.

The authors state that the lack of a therapeutic effect of omega- 3 polyunsaturated fatty acid supplementation in their study is difficult to explain. The observed lack might be partially explained by the short periods and lower dosage of omega-3 polyunsaturated fatty acid supplementation compared with the previously mentioned studies.

A randomized, double-blind, placebo- controlled study on humanpatients with moderate and severe chronic periodontitis investigated the effect of omega-3 polyunsaturated fatty acid supplementation as an adjunct to scaling and root planing (Stando et al., 2020).

At the end of the 12 week period there was as significant reduction in gingival index, sulcus bleeding index, probing pocket depth and clinical attachment level in the test group compared to the control group. However, no statistically significant differences in serum C-

reactive protein levels were found. A recent randomized clinical trial of Stando et al (2020) evaluated the effect of dietary supplementation with omega-3 polyunsaturated fatty acids in 30, otherwise healthy, patients with stage III and IV periodontitis.

Periodontal examination 3 months following initial therapy showed a statistically significant reduction of bleeding on probing and improvement of clinical attachment level in the test group compared with the control group.

The beneficial effects of a supplementation with omega-3 polyunsaturated fatty acids in nonsurgical treatment of periodontitis is also supported by two recently published meta-analyses.

Kruse et al. concluded in their systematic review and meta-analysis that omega-3 polyunsaturated fatty acids seem to have a positive effect on periodontal wound healing or the periodontal parameters clinical attachment level and probing pocket depth. Therefore, patients receiving periodontal treatment might benefit from nutritional counselling.

Similar to that, the meta-analysis of Heo et al (2022) suggests that supplemental or dietary intake of omega-3 polyunsaturated fatty acids for the treatment of periodontitis may have a positive impact on the disease. El-Sharkawy et al (2010) investigated the effect of an adjunctive treatment of chronic periodontitis patients with a combination of omega-3 polyunsaturated fatty acids and low-dose aspirin.

Clinical measurements included plaque index, modified gingival index as well as bleeding on probing, probing pocket depth, and clinical attachment level (Rajasekhar et al., 2004; Cochran 2008; Passoja et al., 2008; Elwakeel et al., 2015). There were no statistically significant differences between test and control groups at different time intervals regarding plaque index and gingival index.

However, there was a significantly greater reduction in probing pocket depth and gain in clinical attachment level in the test group compared with the control group at 3 months and at 6 months postbaseline. There was a statistically significant reduction in RANKL concentrations at 3 and 6 months in the omega-3 plus aspirin group.

The matrix metalloproteinase-8 levels at 3 months were lower in the test group but not statistically significant. However, the matrixmetalloproteinase-8 level at 6 months was statistically significantly lower in the omega-3 plus aspirin group compared with the control group. The significant clinical and biochemical improvements in the test group are imputed to the anti-inflammatory impact of the omega-3 polyunsaturated fatty acids, which is further enhanced by the combination with aspirin. The impact of omega-3 polyunsaturated fatty acids on these biomarkers is assumed to be also mediated via resolvins.

The suggested mechanisms of this impact include the reduction of upstream proinflammatory cytokines directing neutrophils to apoptosis and nonphlogistic recruitment of monocytes. Studies have indicated the inhibition of interleukin-1β and tumor necrosis factor alpha and the reduction of the infiltration of neutrophils into inflamed tissues by esolvins (Serhan et al., 2002; Bannenberg et al., 2005; Arita et al., 2005). Elwakeel et al (2015) investigated the combination of omega-3 polyunsaturated fatty acids and low-dose aspirin as adjunct to nonsurgical periodontal therapy in chronic periodontitis patients with type 2 diabetes.

Elevated levels of interleukin-1β are associated with numerous inflammatory disorders, including periodontitis. A significant reduction of the interleukin- 1β level indicates resolution of inflammation (Elkhouli 2011).

Furthermore, Elwakeel et al. (2002) investigated the impact of the treatment in each group on the glycemic control by measurement of the glycated hemoglobin A1c in fasting venous blood samples. Statistical analyses revealed a significant reduction in probing pocket depth and gain in clinical attachment level at 3 and 6 months in the omega-3 plus aspirin test group compared with control. The hemoglobin A1c levels showed a reduction in both groups with no significant difference. However, the test group showed a significant reduction in levels of interleukin-1β and monocyte chemoattractant protein-3 at 3 and 6 months compared with the placebo control. These were produced as result of the supplementation with omega-3 polyunsaturated fatty acids plus aspirin resulting in inhibition of superoxide production, chemotaxis and migration of polymorphonuclear neutrophils, and reduction of the production of proinflammatory enzymes and cytokines (Kawai et al., 2007; Passoja et al., 2008).

Elkhouli (2011) investigated the effect of the combination of omega- 3 polyunsaturated fatty acids plus aspirin on regeneration of single grade II furcation defects. Patients with at least a single grade II furcation were randomly allocated into two groups. Opposed to interleukin-1β, interleukin-10 is an anti-inflammatory cytokine with immunoregulatory functions, including suppression of interleukin-1 receptor antagonist. At the end of the observation period, there was a statistically significant greater reduction in probing pocket depth and gain in clinical attachment level in the test group compared with the control group. Whereas there was also a significantly greater reduction of the mean interleukin-1β concentrations in the test group, no significant differences between the groups were observed in mean interleukin-10 concentrations.

In their recent randomized clinical trial, Castro dos Santos et al (2020) investigated the clinical and immunological effects of orally administered omega-3 polyunsaturated fatty acids in combination with low-dose aspirin as adjunct to scaling and root planing for the treatment of periodontitis in patients with type 2 diabetes.

The test 1 group also showed clinical attachment level gain in moderate and deep pockets. The levels of interferon-gamma and interleukin-8 decreased over time for both test groups, whereas the interleukin-6 and hemoglobin A1c levels were lower only in the test 1 group.

The authors of this study concluded that the adjunctive use of the omega- 3 polyunsaturated fatty acids and low-dose aspirin combination, administered after periodontal debridement, provides clinical and immunological benefits to the treatment of periodontitis in patients with type 2 diabetes (Castro dos Santos et al., 2020).

Animal studies have shown that animals fed with long-chain omega-3 polyunsaturated fatty acids tend to show an increased rate of bone formation.

Derivatives of the omega-3 and -6 polyunsaturated fatty acids will, depending on the existing omega-6/ omega-3 ratio, induce the differentiation of mesenchymal stem cell precursors into adipocytes or osteoblasts (Watkins et al., 2010). This effect on mesenchymal stem cells favoring or promoting osteoblastogenesis, together with the inhibitory effect on

osteoclastogenesis, may indicate a beneficial effect of omega-3 polyunsaturated fatty acid supplementation regarding maintenance of bone mineral mass (Salari and Abdollahi 2009).

Therefore, the effect of omega-3 polyunsaturated fatty acids on bone metabolism might be a combination of reducing bone resorption and increasing bone formation.

However, different types of omega-3 polyunsaturated fatty acids may have a different impact on bone metabolism. Docosahexaenoic acid in particular seems to act as a key controller of hepatic lipid synthesis and is involved in the suppression of lipogenesis.

Furthermore, since one-third of total circulating interleukin-6 levels are expressed predominantly by adipocytes, reduction in fat mass could contribute to a reduction of interleukin-6 levels, a central player in the regulation of inflammation and capable of inducing insulin resistance (Makki et al., 2013).

Some of them affect areas that not only improve systemic health and general wellbeing but might also have a conceivable impact on periodontal health or periodontal treatment, such as immune response or immune modulation, oxidative stress, bone metabolism or resorption, joint health, and fat metabolism, as well as body weight and body composition. However, caution is advised with eicosapentaenoic acid and docosahexaenoic acid supplementation in patients who are at risk of excessive bleeding or patients on anticoagulant medications. The coagulation status of those patients should be monitored regularly. Although these findings maynot translate to impaired immune responses in vivo, it should be considered in patients with compromised immune systems.

No serious adverse effects have been reported during pregnancy and lactation due to fish oil supplementation (Castro dos Santos et al., 2020).However, considering the already published indications of health benefits due to supplementation with fish oil or omega-3 polyunsaturated fatty acids and the lack of really significant side or adverse effects, supplementation with fish oil or omega-3 polyunsaturated fatty acids can be justified and might even exert positive effects onperiodontal condition or periodontal health.

⇒ **Iron salts influence on periodontitis**

A low-iron diet can cause serious behavioral, functional, and cognitive problems in children, and evidence shows that iron-deficient children of- ten show inferior performance in terms of intelligence and motor functions compared to their normal peers (Pahel et al., 2007). Iron supplements are commonly prescribed to prevent iron deficiency anemia; however, tooth discoloration is a major side effect (Chin et al., 2016). Parents are often concerned about black discoloration of the teeth due to iron drops and commonly seek dental treatment. Many parents mistake iron staining for dental caries or believe that iron supplements have resulted in the development of dental caries and decide to discontinue it. This will lead to early iron deficiency anemia in children, and the adverse consequences may remain for years (Primosch et al., 2001). Insoluble iron compounds react with the gingival crevicular fluid and subgingival bacterial metabolites and produce sulfur-containing compounds, such as hydrogen sulfide, which is responsible for tooth discoloration. Considering the high levels of this metabolite in children with poor oral hygiene or enamel defects, such children are more susceptible to greater tooth discoloration (Szatko et al., 2004). In addition, sweeteners added to the available iron drops in the

Iranian market often contain ferrous sulfate due to their unfavorable taste. These sweeteners may also cause dental caries (Martins-Júnior et al., 2013).

The possible effects of iron regarding caries development, enamel decalcification, saliva viscosity, tooth staining, and oral microbial flora have been extensively investigated, with controversial results (Eskandarian et al., 2013). Shojaipour et al. (2010) indicated black discoloration of teeth due to iron drop consumption. Similar results were reported by Christofides et al. (2006) and Pushoanjali et al. (2004). Ellingsen et al. (1982) and Reid et al. (1977) discussed that ferrous sulfate is an extrinsic factor causing tooth discoloration, whereas Addy and Moran (1985) reported that use of metal salts did not cause tooth discoloration. According to them, the exact mechanism of metal binding salts to tooth surfaces is not clearly understood, and it seems that some superficial changes occur as a result of interactions with the acquired pellicle.

Considering the significance of using iron drops and the gap of information regarding the structural changes of primary enamel following exposure to iron salts, this study aimed to assess the effect of four commonly consumed iron salts on the demineralization and discoloration of primary incisor enamel subjected to artificial cariogenic challenge (ACC) and saline immersion.

1.2.2. Material and methods

1.2.2.1 *Sample Collection and Preparation*

This in vitro experimental study was conducted on sound primary central incisors extracted within the past month due to either severe mobility or over-retention. The sample size was calculated to be 9 in each group, according to a pilot study considering $\alpha = 0.10$, $\beta = 0.25$, and minimum effect size between each two groups to be 1.81. Thus, a total of 90 teeth were enrolled. Teeth with carious lesions, fractures, and enamel structural defects (such as hypoplasia) were excluded. The teeth were stored in saline after extraction, and the saline was refreshed every 48 h.

S. mutans (ATCC35668 and PTCC1683) was purchased in lyophilized form from the Iranian Microbial Culture Collection. The frozen vial was rinsed under lukewarm water to defrost. The bacteria were then transferred to blood agar (Liofilchem, Roseto degli Abruzzi, Italy) and incubated in the presence of CO2 for 18–24 h. Next, the bacteria were transferred to brain heart infusion broth (Merck, Darmstadt, Germany) under a hood.

For preparation of the solution containing 25 mg iron in 3 cc saline, 228.19 mg of ferrous fumarate, 353.21 mg of ferrous ammonium citrate, 373.2 mg of ferrous sulfate, and 647.6 mg of ferrous gluconate (based on the molecular weight of iron salts and atomic number of iron) were required for each tube (Chimi®, Alvand, Iran).

The collected teeth were cleaned with pumice paste and a low-speed handpiece. Next, the root and crown were separated at the cementoenamel junction, and the pulp chamber was sealed with composite resin.

To prepare the artificial cariogenic solution for ACC, 3.7 g of brain heart infusion broth, 0.5 g of extracted yeast (Merck, Germany), 2 g of sucrose (Sigma-Aldrich, Burling- ton, MA, USA), and 1 g of glucose (Sigma) were dissolved in 100 mL of distilled water; 100 μL of freshly cultured standard-stain (ATCC35668) S. mutans (18–24 h) was added to the cariogenic medium, while the pH remained at 4. One test tube was allocated to each specimen.

1.2.2.2 *Grouping*

The total of 90 teeth were randomized into 10 groups (n = 9). The teeth were subjected to ACC in five groups and immersed in saline in the remaining five groups. Each group subjected to ACC or saline was also exposed to one iron salt.

As explained earlier, in order to achieve 25 mg of iron in each test tube (standard amount of iron in commercial iron drops), 373.2 mg of ferrous sulfate, 228.1 mg of ferrous fumarate, 353.21 mg of ferrous ammonium citrate, and 647.6 mg of ferrous gluconate were required. After weighing using a laboratory precision balance type EW-N/EG-N (Kern & Sohns, Hamburg, Germany), the iron salts were added to the test tubes such that in the first five groups, 200 mL of saline was added to each test tube, and 100 μL of the fresh culture was added to the second five groups. The test tubes were then placed in a Bain–Marie shaker (Biotech, Nordost, Germany) at 37 ◦C for 10 min. Next, they were incubated at 37 ◦C for 48 h. To prevent contamination, the tubes were capped. The media were refreshed every 48 h. After 2 weeks, they were removed from the medium and their color was measured and coded using the Vita Shade Guide (Vita Zahnfabrik, Bad Säckingen, Germany), which has 16 color codes from A1 to D4.

The color of the teeth was measured before and after the intervention in a blind manner such that the examiner was not aware of the group allocation of specimens. The teeth were coded as follows:

Score 0: No staining;

Score 1: Slight staining;

Score 2: Heavy staining (out of the range of the Vita Shade Guide).

1.2.2.3 *Structural Assessment*

Two teeth were selected randomly from each group (a total of 20) for the scanning electron microscopic (SEM) assessment. The teeth were dried with warm air, gold sputter-coated, and inspected under a scanning electron microscope. In addition, to assess the amount of iron uptake, energy dispersive X-ray spectroscopy (EDX) was performed, which allows identification of constitutional elements semi-quantitatively.

1.2.2.4 *Statistical Analysis*

Data were analyzed using SPSS version 22 (IBM Inc., Armonk, NY, USA). Normal distribution of data was evaluated by the Kolmogorov–Smirnov test. The color change data were analyzed by the Kruskal–Wallis test, while the iron uptake data were analyzed by one-way ANOVA followed by Tukey's test. The level of significance was considered at $p < 0.05$.

1.2.3. Results

1.2.3.1 *Results of Samples Measurement*

The Kolmogorov–Smirnov test confirmed normal distribution of data regarding the amount of released iron ($p > 0.05$). One-way ANOVA was used to compare the mean amount of iron released from different iron salts, which showed a significant difference in this respect among the groups (Table I.1.1., $p = 0.003$). Thus, pairwise comparisons were performed by

Tukey's test. As shown, the mean amount of released iron in the ferrous ammonium ($p = 0.049$) and ferrous sulfate ($p = 0.003$) groups was significantly higher than that in the control group. The mean amount of released iron in the ferrous sulfate group was also significantly higher than that in ferrous gluconate ($p = 0.008$), ferrous ammonium ($p = 0.054$), and ferrous fumarate ($p = 0.054$) groups. No other significant differences were noted ($p > 0.05$).

One-way ANOVA showed a significant difference in the mean amount of iron released from different iron salts in saline (Table I.1.1., $p = 0.054$). Thus, pairwise comparisons were performed by Tukey's test. As shown, the mean amount of released iron from the ferrous sulfate group was significantly higher than that in the control ($p = 0.057$), ferrous fumarate ($p = 0.083$), ferrous ammonium ($p = 0.099$), and ferrous gluconate ($p = 0.103$) groups.

An independent t-test was used to compare the ACC and saline groups regarding the mean amount of released iron. The mean amount of released iron from ferrous ammonium citrate in the saline medium was significantly higher than that in the cariogenic medium. Statistical analysis indicated that there was a higher iron uptake in the ACC group when compared with saline.

Table I.1.1. Mean amount of iron released from different iron salts in the experimental groups subjected to ACC and saline immersion.

Environment	Group	Mean (SD *)	Min	Max	Fisher's Statistic	*p*-Value
ACC	Control	1.71 (1.2)	0.97	2.64	18.32	0.003
	Ferrous gluconate	6.82 (2.4)	4.23	8.91		
	Ferrous fumarate	8.79 (4.5)	4.16	14.49		
	Ferrous ammonium citrate	13.99 (2.1)	11.58	14.87		
	Ferrous sulfate	25.98 (3.7)	22.68	26.81		
Saline	Control	1.16 (0.8)	0.9	1.62	4.96	0.054
	Ferrous fumarate	2.85 (1.9)	2.10	3.21		
	Ferrous ammonium citrate	3.61 (0.7)	2.95	4.13		
	Ferrous gluconate	3.80 (0.1)	3.85	3.92		
	Ferrous sulfate	17.99 (9.46)	12.41	25.68		

* SD: Standard deviation.

1.2.3.2 *Results of Color Change*

The Kruskal–Wallis test was used to compare the color change of teeth in different groups subjected to ACC. The results showed a significant difference in color change among the five groups subjected to ACC (Table I.1.1.2., $p = 0.61$). The Mann–Whitney test was applied for pairwise comparisons, which showed that the color change in all iron salt groups was significantly higher than that in the control group ($p = 0.083$ for all).

The Kruskal–Wallis test was also used to compare the color change of teeth in different saline groups. The results showed a significant difference in color change among the five saline groups (Table I.1.2., $p = 0.61$). The Mann–Whitney test was applied for pairwise comparisons,

which showed that color change in all iron salt groups was significantly higher than that in the control group (p = 0.083 for all). Pairwise comparisons of the iron salt groups subjected to ACC and immersed in saline by the Mann–Whitney test (Table I.1.3.) showed that the color change in all ACC groups was significantly higher than that in saline groups (Figures I.1.1 and I.1.2) (p = 0.083 for all).

Table I.1.2. *Color change of the teeth in different groups subjected to ACC and saline immersion.*

Environment	Group	Mean Rank	Min	Max	Kruskal-Wallis Statistic	*p*-Value
ACC	Control	1.5	1	3	9.00	0.061
	Ferrous gluconate	6.5	1	8		
	Ferrous fumarate	6.5	2	8		
	Ferrous ammonium citrate	6.5	1	7		
	Ferrous sulfate	6.5	1	8		
Saline	Control	1.5	1	4	9.00	0.061
	Ferrous fumarate	6.5	2	8		
	Ferrous ammonium citrate	6.5	1	9		
	Ferrous gluconate	6.5	2	8		
	Ferrous sulfate	6.5	1	7		

Figure I.1.1. Tooth color change in ACC group.

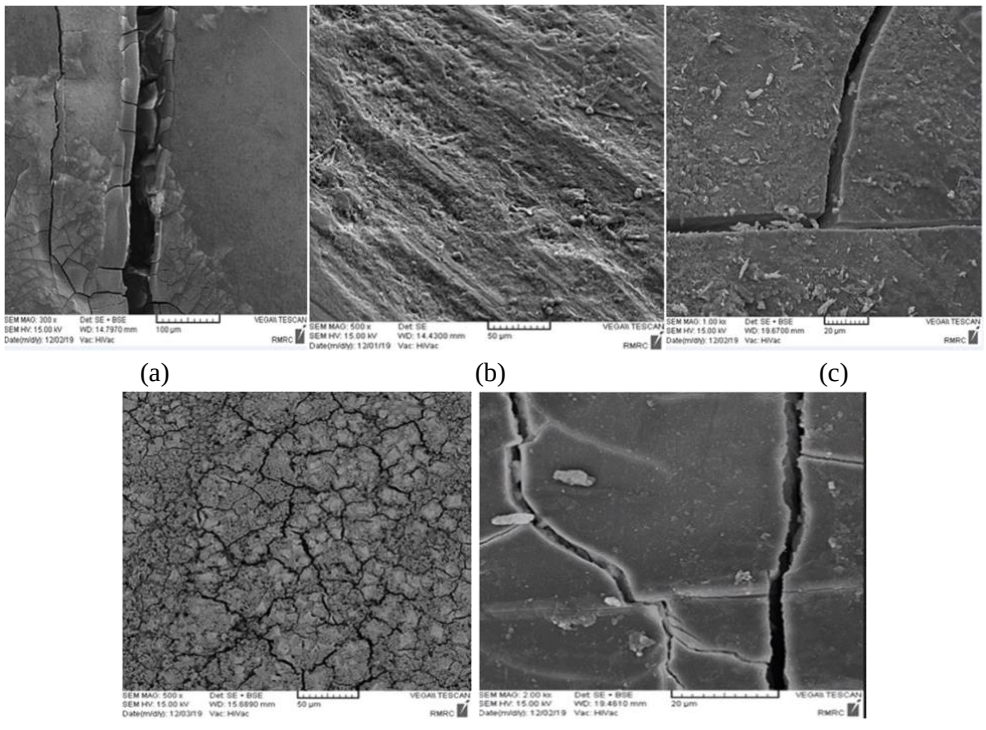
Figure I.1.2. Tooth color change in normal saline group.

Table I.1.1.3. Comparison of the color change of the teeth in different iron salt groups subjected to ACC and saline immersion.

Group	Saline	ACC	Min	Max	Mann-Whitney Statistic	*p*-Value
	Mean Rank	Mean Rank				
Control	1.5	3.5	1	5	0.001	0.083
Ferrous fumarate	1.5	3.5	2	5	0.001	0.083
Ferrous ammonium citrate	1.5	3.5	1	6	0.001	0.083
Ferrous gluconate	1.5	3.5	1	7	0.001	0.083
Ferrous sulfate	1.5	3.5	2	6	0.001	0.083

1.2.3.3 *Structural Assessment*

The SEM micrographs revealed that the number and depth of cracks in all four iron salt groups subjected to ACC were greater than those in the saline groups (Figures I.1.3.and I.1.4.). The number of cracks was the greatest in the ferrous sulfate group, followed by ferrous amomonium citrate, ferrous fumarate, and ferrous gluconate, in decreasing order of fremonium citrate, ferrous fumarate, and ferrous gluconate, in decreasing order of frequency in both media.



(a) (b) (c)

(d) (e)

Figure I.1.3. SEM micrographs of specimens exposed to different iron salts and subjected to ACC: control group, (b) ferrous sulfate group, (c) ferrous ammonium citrate group, (d) ferrous fumarate (**a**) control group, (**b**) ferrous sulfate group, (**c**) ferrous ammonium citrate group, (**d**) ferrous fumarate group, (e) ferrous gluconate group. group, (**e**) ferrous gluconate group.

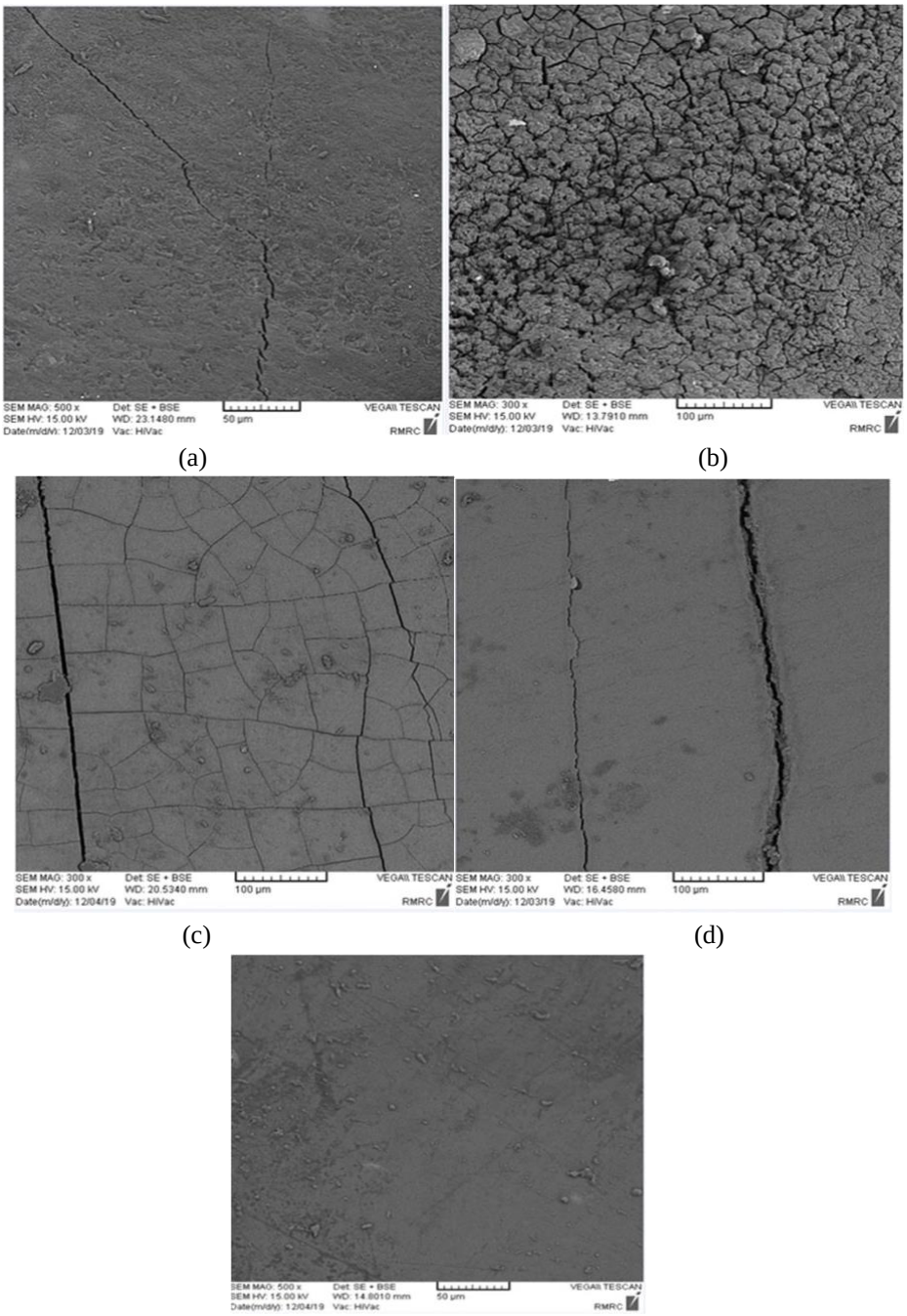


Figure I.1.4. SEM micrographs of specimens exposed to different iron salts in saline: (**a**) control group, (b) ferrous sulfate group, (c) ferrous ammonium citrate group, (d) ferrous fumarate group, (e) ferrous gluconate group.

1.2.4. Discussions

This study assessed the effects of four commonly consumed iron salts on the demineralization and discoloration of primary incisor enamel subjected to ACC and saline immersion. The color change of specimens subjected to ACC was greater than that of those in

saline ($p = 0.083$). The teeth subjected to ACC absorbed greater amounts of iron than did those in saline ($p = 0.023$). SEM assessment revealed a regular pattern of enamel prisms, with some broken prisms and superficial cracks in the teeth immersed in saline. The teeth subjected to ACC showed numerous fractures and cracks, which were greater in ferrous sulfate group.

Similar to the present study, Mehran et al. (2009) reported the effect of iron salts on tooth color. They assessed the atomic absorption of iron, tooth discoloration, and changes in primary enamel structure caused by two different types of iron drops. They reported that teeth exposed to Kharazmi iron drops and ACC experienced greater structural changes and discoloration and had higher iron uptake. Mortazavi et al. (2014) indicated black discoloration of teeth in patients who used iron supplements with high amounts of iodine, and this occurrence had a higher frequency in those with poor oral hygiene. Another study showed that optimal oral hygiene had a preventive effect on tooth discoloration following the use of iron supplements, and fluoride was also suggested for its preventive effect (Eskandarian and Joshan, 2005).

In the present study, the teeth subjected to ACC experienced greater discoloration than those in saline, which can be due to the impaired integrity of enamel after ACC, which would increase the contact area of the enamel with iron ions, subsequently resulting in a greater uptake of iron and higher degree of discoloration. The presence of structural defects, such as hypomineralization, decreases the enamel's resistance to bacterial acid attacks and leads to faster and greater structural degradation, making the enamel more susceptible to discoloration (Szatko et al., 2004).

Iron compounds have metal ions and thus create insoluble brown-black stains, which cause tooth discoloration. In this study, the severity of tooth discoloration was variable among different saline groups. Accordingly, ferrous sulfate caused maximum discoloration, while ferrous fumarate caused minimum discoloration. EDX analyses revealed significantly greater iron uptake by teeth subjected to ferrous sulfate, but the difference among the three other iron salt groups was not significant in terms of tooth discoloration. Tooth discoloration caused by iron is an external discoloration. Iron chemically reacts with the tooth surface and causes its black discoloration (Primosch et al., 2001; Szatko et al., 2004). In addition, the greater the contact of tooth with iron, the greater the iron uptake and the resultant discoloration would be.

SEM assessments have indicated that the prevalence of caries (number and depth of cracks) is correlated with the degree of discoloration and iron uptake. Eskandarian and Joshan (Eskandarian and Joshan, 2005) reported that iron supplementation did not increase the frequency of caries and even decreased the rate of enamel demineralization. The same results were reported by Delbem et al. (2012) and Martinhon et al. (2006). Eshghi et al. (2012) and Ribeiro et al. (2012) found that iron supplementation decreased the rate of caries. Thus, it may be concluded that in carious teeth, enamel porosities, loss of enamel prisms, and the large gaps formed between the enamel prisms would increase the iron uptake and subsequent discoloration. In other words, dental caries leads to greater iron uptake and greater discoloration, and iron supplementation does not lead to caries development. However, some others have reported contrary results. For instance, Tabari et al. (2013) demonstrated a significant reduction in enamel microhardness as the

result of exposure to iron drop. However, they did not mention the name of the iron drop, and this result may have been due to high acidity of the iron drop and subsequent enamel erosion.

The cracking of teeth can also be attributed to factors such as the crystallization behavior of iron salts, tooth dehydration when exposed to air, and replacement of calcium with iron. The four iron salts evaluated in this study have different crystallization behaviors, which may explain the differences in the iron uptake and structure of enamel in the different groups. Structural differences among the groups can also be due to the different pH values of iron drops. Ferrous sulfate is more acidic than the other three, which may explain the greater structural changes in this group. The SEM results revealed a greater depth of cracks in teeth subjected to ACC compared with those immersed in saline, which was expected, considering tooth demineralization in ACC; this finding was in line with the results of Mehran et al. (2009). Iron in very high doses protects the enamel surface from bacterial acid attacks since it forms a hydrous ferric oxide layer on the tooth surface. A previous study showed the high affinity of this layer for calcium and phosphorus in the saliva (Alves et al., 2011). Due to an increase in the calcium and phosphorous content of this layer, the tooth surface is protected against acid attacks and erosion, and remineralization may be induced. However, such cariostatic effects depend on the amount of iron (Alves et al., 2011). Pani et al. (2015) showed that tooth discoloration due to iron exposure was greater compared with exposure to different iron compounds with an equal iron dosage. This finding may be due to the difference in the speed of iron uptake in the form of ferric and ferrous. Ferrous has a faster solubility rate than ferric; thus, it causes less discoloration. The compounds used in the present study had the ferrous form of iron. The solubility of ferrous sulfate is higher than the other three iron salts. Tooth discoloration by iron was significantly greater in ferrous sulfate group in this study. Thus, it may be concluded that the lower the solubility of the iron salt, the lower the release of iron and the lower the discoloration would be. In other words, tooth discoloration depends on the amount of iron ions and free iron in the oral environment.

Although some studies emphasized that silver diamine fluoride solution stains caries black, and the children and their parents must be well informed before application, the influence of diet on staining offers multiple opportunities for future research (Chen et al., 2018).

The manufacturers of iron drops are advised to use formulations that do not release iron into the oral environment. To achieve this goal, the affinity of iron for the elements in the complex should be higher than its affinity for calcium in the tooth structure. In addition, it should not cause any gastrointestinal problems and should be released upon exposure to stomach acid.

Limitations and Suggestions for Future Studies

We emphasize that, according to the little difference in composition observed between saliva and normal saline, it is suggested that future studies add artificial saliva to their experiments. Moreover, items such as vitamin C can be investigated as an addition, due to increased absorption of iron when accompanied with vitamin C.

1.2.5. Final remarks

ACC increased the structural porosities of the teeth and led to greater iron uptake and, consequently, higher discoloration. The maximum structural changes and subsequent staining

occurred in the ferrous sulfate group followed by ferrous ammonium citrate, ferrous fumarate, and ferrous gluconate.

Therefore, it is mandatory to consider implementing a uniform global policy for improving the quality of the iron supplements and the benefit of their considerable effects, although there is no scientific evidence that they play a key role in dental prevention.

In summary, an increasing number of studies have revealed aspects and effects of these so-called lifestyle or fitness supplements and superfoods that may have an impact on periodontal health and healing after treatment. Against the background of periodontal disease as a chronic inflammatory disease involving bone and connective tissue degradation, a deeper insight and understanding of the potential anti-inflammatory effects of supplements and their effects on bone and connective tissue metabolism could help to develop new prevention and treatment strategies. However, some the current evidence is of a very low quality, and more validated scientific data are required before their possible use in prevention or treatment of periodontal diseases can be made.

1.3. Age related factors

1.3.1. Introduction

The most important trend of the 21st century is the general trend of an aging population. The aging of the population is the outcome of several interrelated changes, including falling birthrates, longer life expectancies, and later deaths (Fontana et al., 2014; Partridge et al., 2018). By 2050, it is predicted that around 16% of the world's population will be over the age of 65, which is more than double the present number and a fivefold rise since 1950 (Verma et al., 2021; Qi et al, 2021).

Age-related declines in cognitive abilities such as memory, judgment, language, and focus are a natural consequence of the aging process. Neurodegenerative, vascular, and dysthymia/dysphoria disorders are all potential causes. Social, functional, and vocational activities can all be impacted by impairments in cognition and IQ (Etgen et al., 2011; Campisi et al., 2019).

The brain's microstructure undergoes considerable alterations as a result of aging and diseases, leading to cognitive loss. Changes in brain morphology (the shape and structure of the brain) are a normal part of the aging process, with the most common alteration being significant atrophy (Slade et al., 2014).

The neuroimaging community has extensively examined age- and disease-related changes in the structure of the brain. For the first time, cross-sectional data may be compared directly to form attributes from atlases that are universally acknowledged (Slade et al., 2014). Global brain shrinkage, changes in brain functional responses, and cognitive decline are all common side effects of normal aging (Nyberg and Wåhlin 2020). As a result of this, brain changes exhibit a significant degree of individual variation and appear to be reliant upon different factors, such as mastication (Fischl et al., 2002).

Impaired cognitive function is associated with both nonmodifiable (such as age and gender) and controllable (such as blood pressure and diabetes) risk factors (Beydoun et al., 2014; Gerstorf et al., 2014; Voss et al., 2016). Cognitive impairment is not an illness but a description of a condition. It means that the person in question has trouble with tasks such as memory or paying attention. They might have trou- ble speaking or understanding. Additionally, they might have difficulty recognizing people, places or things, and might find new places or situations overwhelming. Despite extensive research, no definitive treatment for this cognitive deficit has yet been found (De Brujin et al., 2014). Since more people with mild cognitive impairment than without it go on to acquire Alzheimer's disease or similar dementia conditions, researchers have tried to examine and prevent mild cognitive impairment in an effort to diminish the societal and financial costs related to the condition. Potentially modifiable risk factors for cognitive impairment have been identified as poor dental health and poor mastication (Fotuhi et al., 2009).

According to scientific evidence, frequent sensory input when chewing causes an increase in blood flow to the brain and a greater number of pyramidal neurons in the hippocampus. When it comes to humans, this area of the brain is critical for the generation and retrieval of episodic memory (Henke et al., 2010; Reyes-Ortiz et al., 2013). Neurotransmitter function may be negatively affected by insufficient mastication capacity as well as by the absence of afferent stimulation by masticatory receptors. This may result in a decrease in the amount of acetylcholine produced, which is responsible for the stimulation of electrical flow between neurons (Tulving and Markowitsch 1998; Batty et al., 2013).

Poor oral health has been associated with cognitive impairment in several long-term cohort studies (Onozuka et al., 1999; Makiura et al., 2000). A correlation has been shown between the number of teeth in a person's mouth and their level of cognitive performance. It has also been shown in several case studies that restoring tooth and masticatory function with an appropriate prosthesis can increase functional activity in the brain (Terasawa et al., 2002; Jiang et al., 2011). The periodontal ligament and masticatory muscle are thought to receive their nerve supply from the trigeminal nerve. The attenuation of trigeminal nerve sensory input as a result of ongoing tooth loss has been demonstrated to impair higher-level brain functions including learning and memory. (Watanabe et al., 2002; De Marchi et al., 2012). Improvements in oral motor performance and shifts in mandibular position are closely connected to deterioration in masticatory muscle function, and degradation of the α'-γ coupling mechanism may be associated with senile dementia in some cases (Peres et al., 2015). Previous research has shown a link between dental health and dementia, but the pathogenic processes by which this occurs remain unclear.

Despite the fact that we are aware of certain data that suggest otherwise, we are not aware of any meaningful evidence about the impact that dentures play in the cognitive state of older people who are edentate.

It is becoming increasingly clear that oral health may play a crucial role in a person's cognitive performance as they age. Many studies have found a link between the number of natural teeth a person has and their cognitive abilities (Hirai and Koshino 2006; Walker et al., 2017). Even if we are aware of certain statistics that suggest otherwise, we are not aware of any

meaningful evidence about the impact that dentures play on the cognitive state of older people who have lost all of their teeth.

Although preliminary clinical investigations have supported this logic, it is still just conjectured as to whether or not it is possible to reverse decrease in cognitive function by improving chewing performance through restorative treatments. As a result, the present study was designed to test the hypothesis that dentures, acting through the mastication route, will have an impact on the cognitive state of the senior elderly population.

1.3.2. Materials and Methods

1.3.2.1 *Study Population*

The study was conducted with the approval of the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy of Iasi (No. 18/05.05.2022), and the included participants all consented to the procedures. The research was conducted from May 2022 to October 2022. At the recruitment stage, the study objectives were explained, inviting all adults aged 60 years and above to participate in the study. The exclusion criteria were: (1) younger than 60 years old, and (2) cognitive disease already being treated.

Patients seeking treatment at the Faculty of Dental Medicine in Iasi, Romania, were eligible for enrolment; a total of 112 patients who agreed to take part in this study were included.

The intraoral examination was performed by a single examiner who only considered the number of missing teeth and not the efficacy of treatment for determining the edentulousness type. The patients were given free rein as to how they wanted their mastication assessed, and the goal was to determine whether or not cognitive impairment was inversely proportionate to the number of patients with at least some of their original teeth.

1.3.2.2 *Cognitive Dysfunction Assessment*

Cognitive dysfunction was assessed using the Mini-Mental State Examination (MMSE), which is a commonly used tool for measuring cognitive function. The MMSE works well as a screening tool to distinguish between patients with and without cognitive impairment. Since its initial publication in 1975, Folstein's study has been cited about 50,000 times in the Scopus database (Okamoto et al., 2015). Its rapid implementation and widespread use may have contributed to this effect. Furthermore, a recent meta-analysis demonstrated that the instrument's sensitivity was 85%, with specificity around 90%, for the diagnosis of dementia in both community and primary care settings (Folstein et al., 1975).

The instrument can also measure changes in cognitive status that may benefit from intervention when administered repeatedly. The measure should not take the place of a thorough clinical evaluation of mental status, however, as it is unable to diagnose the circumstances surrounding changes in cognitive function. The test also significantly em- phasizes verbal response, reading, and writing.

The Mini-Mental State Examination (MMSE) is used for conducting a complete and methodical evaluation of mental status. The MMSE was translated and validated in Romania (Folstein et al., 2013). Five cognitive processes are examined in this 11-question test: orientation,

registration, atten- tion and calculation, recall, and language. The maximum score achievable is 30. Cognitive impairment is indicated by a score of 23 or less. The MMSE can be administered in just 5–10 min, making it convenient to use frequently and on a regular basis.

1.3.2.3 *Assessment of Covariates*

People aged 60 and above have varying degrees of cognitive impairment. There is a wide variety of potential causes, and often these factors overlap. Recent epidemiological studies estimate that between 4.7% and 8.7% of the older population may have dementia, while as many as 42% may be living with moderate cognitive impairment (MCI) (Sachdev et al., 2015; Creavin et al, 2016; Prince et al., 2022). Controlling for demographic covariates such as age, education, race, and neighborhood (or place of residence) will strengthen the study design.

Screening tests are advised for the diagnosis of cognitive impairment in persons who have a high suspicion of having Alzheimer's disease (AD) or other disorders. Therefore, we collected information about participants' sociodemographic characteristics (i.e., age, gender, education level, place of residence) and health conditions (e.g., presence of chronic conditions).

1.3.2.4 *Statistical Analysis*

The acquired data were examined with IBM SPSS 26.0 (SPSS Inc. Chicago, IL, USA). The statistical significance was set at $p = 0.05$. The descriptive study of the group's general characteristics was reported as frequencies, means, and standard deviations. We employed the Student's t-test and the ANOVA test for comparisons. Correlations between MMSE scores and specific variables were determined by applying linear regression (sex, education, edentulous treatment, and masticatory efficiency).

1.3.3. Results

In total, 108 subjects participated in the study, with an average age of 67.79 ± 14.44 (minimum age of 28 and maximum age of 87) and a greater proportion of female subjects (57.4%), with 53.7% having a high school education, and the majority coming from an urban environment (64.8%). A total of 85.2% of the patients reported comorbidities, with cardiovascular, metabolic, and locomotor problems being the most prevalent (Table I.1.3.).

Concerning dentition-related traits, more than half of the participants had several types of edentation (51.9%), followed by those with complete edentation (35.2%). Only 50% of the edentulous participants underwent prosthetic treatments, 25.9% of them with removable dentures and 24.1% having fixed and removable prostheses. Only 46.3% of the individuals demonstrated adequate masticatory efficiency (Table I.1.3.).

In Table I.1.4., the distribution of the participants' answers to the questions of the MMSE questionnaire is presented. The average value of the MMSE score is 21.81 ± 3.872 out of a maximum value of 30. The increased frequency of answers with low scores can be observed in the case of subjects who have teeth not treated with prosthodontic treatment; thus, in the case where the subject had to count backwards from 100 by decreasing by 7, it was observed that 22% of those who did not have prostheses did not have the ability to achieve this, followed by 62% who managed to do this to a small extent.

Table I.1.3. Characteristics of the study participants.

		No.	**%**
Age	67.79 year (SD 14.44), (min. 28 years, max. 87 years)		
Sex	Female	62	57.4
	Male	46	42.6
Education level	Elementary school	38	35.2
	Secondary school	58	53.7
	University studies	12	11.1
Place of residence	Urban	70	64.8
	Rural	38	35.2
Type of edentation	Partially extended edentulism	14	13.0
	Total edentulism	38	35.2
	Combined edentulism	56	51.9
Treatment of dental edentation	Yes	54	50.0
	No	54	50.0
Type of treatment of dental edentation	Untreated edentulism	54	50.0
	Removable prosthesis	28	25.9
	Composite prosthesis	26	24.1
Subjective masticatory efficiency	Adequate mastication	50	46.3
	Inadequate mastication	58	53.7
Comorbidities	Yes	92	85.2
	No	16	14.8

More than half of the non-wearer subjects had reduced ability to recall the names of three previously heard objects as well as to write a phrase with a subject and a predicate (q 5: score 1–51.9%, q 6: score 0–51.9%). Reproducing a drawing was another situation in which 55.6% of the non-wearer subjects encountered difficulties. Differences between groups of wearer/non-wearer subjects were statistically significant for most of the questions in the questionnaire (Table I.1.4.).

The average value of the MMSE score was 21.81 (SD 3.872) and was associated with edentation treatment ($p = 0.000$), subjective masticatory efficiency ($p = 0.000$), and detected comorbidities ($p = 0.000$). There were no associations between the MMSE and gender distribution, education level, or place of origin (Table I.1.5.).

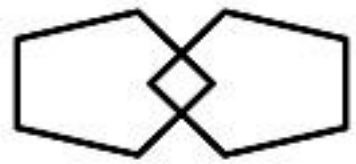
Regarding the MMSE forms, depending on the general characteristics, the statistical analysis indicates that female subjects present a higher frequency of moderate MMSE form scores (35.5%), regardless of the level of education, and the mildest MMSE form scores. More subjects with university degrees (75%) and those from the urban environment present more cases of moderate MMSE than those from the rural environment (34.3%).

Statistically significant differences were recorded in the case of the edentulous treatment variables, where non-wearer subjects presented more moderate MMSE (63%, $p = 0.000$), as well as in the case of subjects who declared that they had ineffective mastication (58.6%, $p = 0.000$) and in the case of those who have comorbidities (34.1%, $p = 0.000$) (Table I.1.5.).

Linear regression analysis (Table I.1.6.) showed that in the case of the association of the MMSE score and the edentation treatment, the correlation coefficient is positive ($B = 3.986$, $p = 0.004$),

which indicates that individuals with a high MMSE score (close to wave max. 30) Partial Regression Plot have prosthodontic treatment. This relationship is also highlighted in Figure I.1.5. where the regression line is positive and the points are grouped in quadrants I and III, demonstrating a tight, positive, and balanced relationship between the two elements.

Table I.1.4. Distribution of participants' answers to the questions of the MMSE questionnaire.

Questions	**Mean ± SD Maxim Value**	**MMSE Scores**	**Edentulism Treatment**		***p***
			No	**Yes**	
1. Orientation: Which (year), (season), (day of the week), (date), (month) is it?	4.15 ± 0.873 Max. 5	2 3 4 5	18.5% 7.4% 63.0% 11.1%	0.0% 0.0% 37.0% 63.0%	0.000
Where are we—(country), (town), (district), (hospital), (floor)?	4.35 ± 0.701 Max. 5	3 4 5	25.9% 51.9% 22.2%	0.0% 25.9% 74.1%	0.000
2.Memory: Say the names of three unrelated objects loudly and clearly, with one-second pauses between them. Ask the patient to repeat all three (1 point for each correct answer). If it does not work the first time, repeat the test until the patient repeats all three words (try up to 5 times). If the patient cannot learn them all, immediate memory cannot be properly assessed	2.28 ± 0.653 Max. 3	1 2 3	7.4% 59.3% 33.3%	14.8% 40.7% 44.4%	0.133
3. Attention and calculation Subtract 7 from 100, then repeat from the result. Continue five times: 100, 93, 86, 79, 72, 65	2.65 ± 1.328 Max. 5	0 1 2 3 4 5	22.2% 3.7% 33.3% 29.6% 7.4% 3.7%	0.0% 0.0% 25.9% 40.7% 18.5% 14.8%	0.001
4. Recall Ask for the names of the three objects learned earlier (1 point for each correct answer).	1.98 ± 0.875 Max. 3	1 2 3	51.9% 18.5% 29.6%	25.9% 29.6% 44.4%	0.022
5. Language: "Make up and write a sentence about anything." (This sentence must contain a noun and a verb.)	1 ± 0.820 Max. 2	0 1 2	51.9% 29.6% 18.5%	14.8% 37.0% 48.1%	0.000
Repeat "No ifs, and or buts."	1 ± 0.00 Max. 1	1	100%	100%	-
Follow a 3-stage command: "Take the paper in your right hand, fold it in half, and put it on the floor." (The examiner gives the patient a piece of blank paper.) Score 1 for each stage.	2.41 ± 0.737 Max. 3	1 2 3	18.5% 33.3% 48.1%	11.1% 25.9% 63.0%	0.277
Read and obey the following: "Please read this and do what it says." (Written instruction is "Close your eyes.")	0.85 ± 0.35 Max. 1	0 1	29.6% 70.4%	0.0% 100%	0.000
Name a pencil and watch.	0.94 ± 0.23 Max. 1	0 1	11.1% 88.9%	0.0% 100%	0.012
 6. Copying:	0.52 ± 0.50 Max. 1	0 1	55.6% 44.4%	40.7% 59.3%	0.177

SCOR MMSE	21.81 ± 3.872	16	22.2%	0.0%	0.000
	Max. 30	17	3.7%	0.0%	
		18	14.8%	0.0%	
		19	33.3%	0.0%	
		21	3.7%	11.1%	
		22	18.5%	0.0%	
		23	0.0%	40.7%	
		24	0.0%	11.1%	
		25	0.0%	14.8%	
		27	0.0%	7.4%	
		29	3.7%	0.0%	
		30	0.0%	14.8%	

Table I.1.5. Cognitive impairment: means values for general characteristics, edentulism treatment, and masticatory function.

		Cognitive Impairment						
		Mean	**SD**	***p* ***	**No MMSE %**	**MMSE Mild Form %**	**MMSE Moderate Form %**	***p* ****
Sex	Female	21.10	3.696	0.025	6.5	58.1	35.5	0.565
	Male	22.78	3.932		8.7	65.2	26.1	
Education level	Elementary school	21.16	3.309	0.184	0.0	65.8	34.2	0.091
	Secondary school	21.90	4.233		10.3	55.2	34.5	
	University studies	23.50	3.398		16.7	75.0	8.3	
Place of residence	Urban	22.09	3.907	0.326	8.6	57.1	34.3	0.503
	Rural	21.32	3.807		5.3	68.4	26.3	
Treatment of dental edentation	No	19.11	2.820	0.000	0.0	37.0	63.0	0.000 *
	Yes	24.52	2.725		14.8	85.2	0.0	
Subjective masticatory efficiency	Adequate mastication	24.68	2.736	0.000	16.0	84.0	0.0	0.000 *
	Inadequate mastication	19.34	2.881		0.0	41.4	58.6	
Comorbidities	No	24.60	5.051	0.000	40.0	40.0	20.0	0.000 *
	Yes	21.18	3.268		0.0	65.9	34.1	

* t test; ** Chi-square test.

A negative relationship was detected between the MMSE scores and subjective masticatory efficiency, which indicates that a decrease in the MMSE score is accompanied by a decrease in subjectively evaluated masticatory efficiency in the study participants (B = 1.513, p = 0.268) (Table I.1.6, Figure I.1.6)

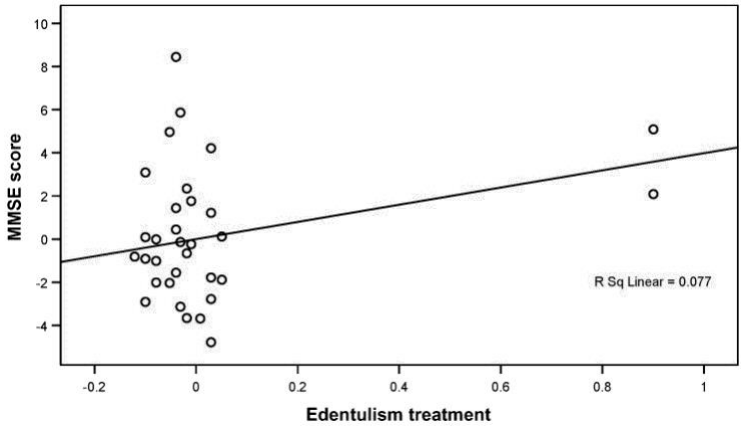


Figure I.1.5. Linear regression plot showing a positive correlation between MMSE scores and edentulous treatments.

Table I.1.6. Linear regression analysis: association between MMSE score, sex, education, dentures, and subjective masticatory efficiency.

Dependent Variable	**Independent Variables**				**95% CI for B**		***p***
		B	**SE B**	**Beta**	**Lower Bound**	**Upper Bound**	
MMSE	(Constant)	18.022	3.011		12.051	23.994	0.000
	Sex	1.887	0.511	0.242	0.874	2.899	0.000
	Education	0.815	0.393	0.135	0.036	1.595	0.041
	Edentulism treatment	3.986	1.359	0.517	1.291	6.681	0.004
	Subjective masticatory efficiency	−1.513	1.360	−0.196	−4.210	1.184	0.268

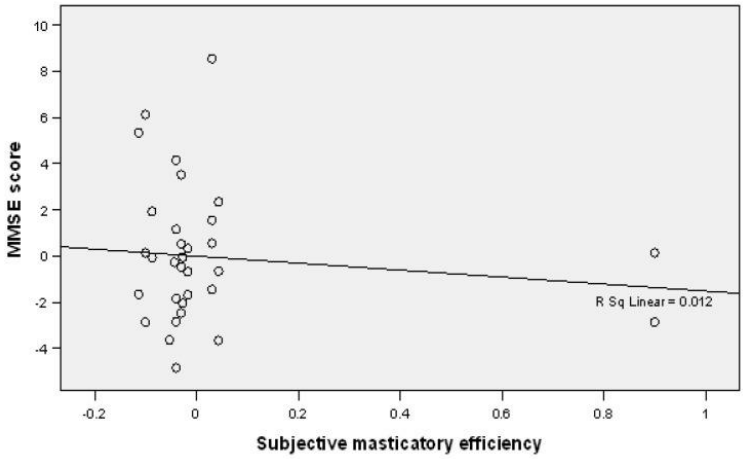


Figure I.1.6. Linear regression plot showing a negative correlation between MMSE scores and subjective masticatory efficiency

1.3.4. Discussions

According to recent findings in oral health and geriatric medicine, a new dimen- sion has emerged in the study of significant links between impaired oral function, oc- clusal/mastication,

and specific systemic illnesses such as cognitive and brain functions. Geriatric syndromes, such as memory and cognitive impairments and dementia, can lead to a steady deterioration, which is sometimes accompanied by other comorbidities (Fukushima-Nakayama et al., 2017; Kumar 2017).

Cognitive issues are typically more prevalent and disabling in older people as a result of their advanced age. According to the findings of our study, older age was substantially related to the onset of cognitive impairment. This finding was in line with our expectations. It has been widely debated in the literature (Peyron et al., 2017) whether there is a correlation between one's socioeconomic position and access to dental treatment, and this does appear to be an essential role in cognitive impairment. In our study, likely due to the limited number of subjects, we did not find any link between the patients' socioeconomic status and cognitive impairment.

According to several studies, the existence of natural teeth in humans appears to be linked to higher cognitive performance (Bergdahl et al., 2007; Galindo-Moreno et al., 2021). After conducting a literature review on the relationship between occlusion and human brain function, Okamoto and his colleagues in 2010 came to the conclusion that "mastication and other movements stimulate activity in the cerebral cortex and could be useful in avoiding degeneration of cognitive ability. It has been hypothesized that rhythmic chewing motions, which enhance blood flow in the brain and stimulate various sections of the cortex, are responsible for this phenomenon and that an increase in blood oxygen levels in the prefrontal cortex, as well as in the hippocampus, may influence learning and memory function (Onozuka et al., 2002; Hirano et al., 2008).

Losses in masticatory function, rather than number of teeth, has been found to have a significant effect on cognitive performance (Fontijn-Tekamp et al., 2000). In this context, it is well known that the molars are the teeth that can withstand a greater amount of masticatory power and are the primary determinants of masticatory efficiency (Ikebe et al., 2018), and this is true for both natural and artificial occlusion. Therefore, masticatory performance can have a favourable influence on cognitive function (Lopez-Chaichio et al., 2021), regardless of whether it is performed with natural teeth or with prosthetic therapy. The results of our study are similar to those of previous studies in the sense that persons with edentulous arches have reduced masticatory efficiency and low MMSE values.

Even more intriguing is the finding that the only significant link between cognitive deterioration and tooth loss was discovered when molars were missing from the mouth after each kind of lost tooth was examined separately. This may be transmitted through the locus coeruleus, which is triggered by a variety of factors including periodontal fibers and proprioceptive jaw muscle spindles (Cerutti-Kopplin et al., 2016). For our study, this could be considered one limitation because we conducted the analysis taking into consideration only the type of edentation and not the type of the remaining teeth.

It is undeniable that tooth loss has been associated with the development of memory and cognitive impairment as well as dementia. Evidence suggests that having fewer than 20 teeth

increases the likelihood of cognitive impairment and dementia in the elderly (Shimazaki et al., 2001; Okamoto et al., 2017).

The findings of Shimazaki et al. revealed that around 50% of the entirely edentulous and 35% of the partially edentulous who did not wear dentures acquired over time a considerable risk of physical handicap and death (Shimazaki et al., 2000; Avivi-Arber and Sessle 2018).

Therefore, preserving as many natural teeth as possible or wearing dentures that are well fitted can be a vital precaution for the oral and physical health of the elderly, especially the more vulnerable population (Takata et al., 2008).

Greater chewing capacity from more functional tooth units on dental occlusion may lead to extended life expectancy; similarly, a greater selection of nutrients in daily meals is similar to intellectual and social activities for a higher functional quality of life (Utsugi et al., 2014). According to the findings of recent investigations, age-related oral deafferentation and age-related changes in brain activity might result in cross-modal problems, such as loss of the ability to taste and smell food. Because of animal research in which hard food was used as feed, the relationship between oral deafferentation and the neurocognitive and neurogenic brain axis was further established (Kumar et al., 2018).

Consequently, there has been a rise in the belief that dental deafferentation and brain aging are linked, which might lead to new treatments for cognitive decline and neurodegenerative illness in the elderly. In both humans and animals, the reduction in hippocampal brain-derived neurotrophic factor levels is linked to the deterioration of brain and masticatory processes. At the same time, the number of dendritic spines in molar-less mice with the reduced distinction of newly neuronal resulted cells is inhibited, which may be associated with damage in hippocampus-dependent spatial memory, a decrease in the growth and survival of new-born cells in the dentate gyrus, an increase in hippocampal amyloid-beta, and a deterioration of norepinephrine neurons in the locus coeruleus (Kumar et al., 2018).

Despite the fact that our brain is in a permanent state of flux, new connections are always being formed, which might result in the acquisition of new abilities or adjustment to a new oral environment. More research is needed to determine if the concept of "neuroplastic- ity" is correct. Studies have also demonstrated the distinctive and reversible neuroplasticity of corticomotor excitability in the context of controlling peri-oral tongue muscles during movements. Two weeks following tongue training, it has been demonstrated that the plastic alterations returned to baseline levels.

A study by Kumar et al. found that in their study group, denture users' cerebral activity returned to baseline levels three months following the placement of new dentures, which was similar to the results of previous research. This is a return to the starting point (Kumar et al., 2018). It is possible that cortical modifications were more "elastic" (i.e., reversible) than "plastic" once the training was discontinued, giving the appearance that the changes were more permanent (i.e., irreversible). The correlation between tooth loss and decreased cognitive performance is supported by the findings of this study. As a result, it is likely that by increasing the efforts that are committed to preventing tooth loss in the adult population it will be possible to achieve the desired results.

It is important to note that this evaluation does have certain limitations. As a result of the small sample size, we were unable to obtain reliable estimations of the parameters governing the study's validity. We also acknowledge that our participants were sampled from one area of the city of Iasi, which may raise questions regarding the generalizability of our results; a future population study could me more randomized, apart from age and presence/absence of teeth.

1.3.5. Final remarks

These findings further highlight the positive impact of periodontal medicine and preserving natural teeth on memory. Beyond the financial repercussions, the true cost of cognitive decline, if we define it as memory impairment as well as personal experiences and relationships, is unquantifiable.

As a future perspective for this pilot study, one might convene a multicenter study group, representative for at least a region of Romania and presenting a possible corelation between patients' cognitive state of mind, prosthetic condition, and quality of life.

Furthermore, it may be possible to retain and safeguard other aspects of a person's wellbeing that cannot be quantified by preventing tooth loss, such as the capacity to live a comfortable life, the conservation of memories, and the maintenance of a sense of one's own personality.

1.4. Systemic disease and periodontitis

1.4.1. Connection between diabetes and periodontal disease

1.4.1.1 *Introduction*

Periodontal disease is the result of chronic inflammatory manifestations, determined by the supra-gingival and sub-gingival accumulation of pathogenic biofilm. The inflammatory host response, with the loss of balance between bacterial aggression and the impaired ability of the immune system to cope with this aggression, leads to different forms of periodontal disease, from gingivitis to severe periodontitis (Jepsen et al., 2017). The reported worldwide prevalence of periodontal disease varies from 45 to 50% in adults (superficial tissue impairment), to over 60% in elderly patients (White et al., 2012; Eke et al., 2016). An important percentage of patients (around 11%) is affected by severe periodontal tissue breakdown (Kassebaum et al., 2014), which can generate different forms of complications, ranging from loss of teeth to an affected quality of life (Buset et al., 2016). Data from the literature also suggest that severe periodontal inflammation is linked to mortality (Soder et al., 2015).

Diabetes mellitus (DM) is a disease with an increased incidence worldwide; the number of patients under 14 years old diagnosed with insulin-dependent diabetes exceeds 500,000, while the number of adult diabetes patients is around 415 million. Moreover, it is estimated that there are about 193 million undiagnosed patients and 318 million people with an increased risk of developing DM during their lifetime (Diabetes care 2017). It is estimated that, if this ascending trend is not stopped, by 2040 there will be 642 million people affected by diabetes (Diabetes Atlas, 2015).

The interrelation between periodontitis and diabetes was intensively studied; increased periodontal inflammation was associated with high serum levels of glycated hemoglobin (HbA1c) in subjects with diabetes and, interestingly, also in subjects without diabetes. It was observed that severe periodontal breakdown increases the risk of diabetes complications (vascular pathology, renal dysfunction) (Borgnakke et al., 2013). Furthermore, severe periodontal breakdown was associated with metabolic syndrome and increased oxidative stress in patients with type 2 DM (Allen et al., 2011). Therefore, it was concluded that severe local inflammation (such as periodontitis) might exert an important role in the etiopathogenesis of diabetes and its complications (Morita et al., 2012), thus patients with diabetes and periodontal disease are more demanding regarding treatment options and efficacy.

The potential effect of non-surgical periodontal treatment (scaling and root planning—SRP) in periodontitis and DM subjects was considered. A systematic review observed a 0.36% reduction in HbA1c at 3 months after SRP (Engebretson and Kocher 2013).

The term endo-periodontal lesion appeared decades ago in order to describe a specific disease that affects the pulp and periodontal tissues simultaneously. Patients with diabetes are more prone to oral infections and periradicular lesions caused by changes in the immune system, qualitative and quantitative changes in the normal flora of the oral cavity and poor peripheral blood irrigation (Al-Fouzan 2014).

Furthermore, endo-periodontal lesions can be a risk factor for severe complications, such as odontogenic sinusitis. Inflammation and/or disruption of the Schneider membrane results in the inflammation of the mucosa and impaired mucociliary function in the maxillary sinus. Impaired mucociliary function results in altered mucus transport, impaired mucosal defense, blockage of sinus ostia and consecutive inflammatory processes (Gamba 2016).

Considering that diabetes adversely affects blood circulation or causes ischemia, sometimes necrotic tissue phenomena may occur (Dhoum et al., 2018). The possible connection between chronic oral inflammatory processes, such as apical periodontitis, endodontic status and systemic health, represents one of the most interesting aspects faced by the medical and dental scientific community; in order to achieve a proper healing potential, all parameters must be stabilized, in our case, inflammation and infection status in a diabetic field.

Glycemic control is essential to prevent diabetes-related morbidity and mortality. Increased glycated hemoglobin A1c (HbA1c) has been linked to diabetic microvascular and macrovascular complications and decreased HbA1c leads to reduced morbidity and mortality (Khaw et al., 2001).

Melatonin is a ubiquitous hormone which can also be found in the oral cavity. The salivary level of melatonin is about 1/4 to 1/3 of the serum level, varying from 1 to 5 pg/mL in daytime to 50 pg/mL during the night (Laakso et al., 1990). The presence of melatonin in saliva is considered to be the result of the passive passage in the salivary glands cells of serum unbound melatonin. Nevertheless, a study found the expression of arylalkylamine-N-acetyl-transferase (an enzyme involved in the night/day rhythmic production of melatonin) in the salivary glands of murine experimental models and human subjects (Shimozuma et al., 2011). The literature data confirm the presence of melatonin receptors in different oral sites, such as epithelial cells,

osteoblasts, and fibroblasts (Cutando et al., 2011), but the role of melatonin in the tissues of the oral cavity remains unclear.

Several studies have investigated the possible effects of melatonin on periodontitis subjects. A study observed the levels in gingival crevicular fluid and saliva in 70 patients with different periodontal pathologies. The researchers observed that the melatonin levels in severe periodontitis subjects were significantly lower than those in healthy or gingivitis subjects (Almiughrabi et al., 2013). Regarding the influence of periodontal treatment, scaling and root planing exerted a positive effect by increasing the salivary melatonin levels in periodontitis subjects (Bertl et al., 2013). However, until now there has been no information regarding the improvement in the severe periodontal status of diabetic patients after non-surgical periodontal treatment and melatonin supplementation in the literature.

The drug supplementation of conventional etiologic periodontal treatment has been widely investigated, with a main focus on antibiotics and anti-inflammatory drugs. Nowadays, there is increased interest in less standardized drugs (such as sub-antimicrobial doses of doxycycline or omega-3 fatty acids), which could generate fewer adverse events and important local and systemic benefits. One of such therapies might also include melatonin intake. We hypothesized that melatonin supplementation, adjunctive to conventional non-surgical periodontal therapy, could improve the clinical periodontal status and decrease the HbA1c in patients with diabetes mellitus and periodontitis and prompt additional benefits to patients with severe periodontal disease.

Doxycycline is an inexpensive, well-tolerated, broad-spectrum antibiotic that has the added benefit of being a potent inhibitor of host-derived matrix metalloproteinases (MMPs), even at subantimicrobial doses. Levels of MMP-class enzymes, including MMP-9 and MMP-8, have repeatedly been shown to be decreased in gingival tissues and periodontal lesions by subantimicrobial doses of doxycycline. Doxycycline in subantimicrobial doses (SDD) (Periostat™, CollaGenex Pharmaceuticals, Inc.; now Galderma R&D) has been approved as an adjuvant to root planning and scaling for the treatment of periodontitis (Caton and Ryan 2011). The additional benefit of conventional subgingival debridement generated by doxycycline in subantimicrobial doses is due to the strong inhibition of extracellular matrix degradation, even in severe cases of periodontitis. The FDA (Food and Drug Administration) approved dose for subantimicrobial doxycycline is 20 mg twice daily for up to 9 months. Antimicrobial action and side effects of antibiotics (for example, the emergence of antibiotic-resistant bacteria) do not occur at the recommended therapeutic doses (Engebretson and Hey-Hadav 2011).

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. We have also assessed the effects of scaling and root planing plus adjunctive systemic treatment with melatonin on periodontal parameters and glycemic control (HbA1c) in patients with type 2 diabetes and chronic periodontitis.

1.4.1.2 *Materials and methods*

⇒ First study

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), 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 reacted with anti-HbA1c antibodies, with the formation of soluble antigen-antibody complexes. In the second step, polyhaptenes were added, which reacted with excess anti-HbA1c, by forming antibody-polyhaptene complexes, which were determined by immunoturbidimetry. The total hemoglobin concentration was determined in a separate channel. In the hemolyzed blood sample, the released hemoglobin was converted into a derivative, with a characteristic absorption spectrum; it was measured in two colors.

The percentage calculation of glycated hemoglobin was 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 was applied:

$$\% \text{ HbA1c} = (\text{HbA1c/Hb}) \times 91.5 + 2.15$$

This evaluation was performed at the beginning of the study and 3, 6 and 12 months after baseline.

Table I.1.7. 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 (55.00%)	29 (56.86%)

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.

⇒ Second study

Inclusion and Design Criteria. In this double-blind, placebo-controlled, single-center study, 74 patients with type 2 diabetes with symptoms of periodontal disease were recruited. Subjects with fastening blood glucose levels higher than 126 mg/dL and glycated hemoglobin higher than 6.5% were defined as diabetic (Mahan et al., 2012).

The study methodology was in accordance with ethical principles, including the Declaration of Helsinki from 2008; the study was conducted under the approval of the University of Medicine and Pharmacy "Grigore T. Popa" Iasi Ethics Committee nr. 30.07.2020. The subjects were informed regarding the study methodology and signed informed consent regarding their inclusion in the study.

The recruited subjects were thoroughly examined in order to assess the presence of periodontitis. Twenty patients out of 74 were excluded at baseline. Subjects who had undergone periodontal or anti-inflammatory treatment in the last 6 months, insulin treatment, significant change in drug use, and treatment of their diabetes or diet were excluded from the study. Other exclusion criteria were pregnancy, lactation, and smoking.

When performing a power analysis on the two analyzed groups with 90% power for a 1% difference in HbA1c between the two groups, a type I error of 0.05, and a type II error of 0.1, a total of 21 patients per group was needed. To anticipate the withdrawal rate of patients, an extra 25% was added to each group, thus the final patient number was 27 patients per group.

In conclusion, 54 subjects were randomly assigned to the study group ($n = 27$) or control group ($n = 27$) by a randomized block procedure.

Anthropometric Measurements. Anthropometric measurements, including height, weight, body mass index (BMI), waist circumference (WC), hip circumference (HC), and waist-to-hip ratio (WHR), were measured by a specialized nutritionist. The Nutritionist 4 software was used for the diet evaluation.

The intensity of physical activity was self-reported by questionnaire. We considered light activity to be 0–600 min/week (coefficient 3.3), moderate activity to be 600–3000 min/week (coefficient 4), and heavy activity to be >600 min/week (coefficient 8) (Gh, 2011).

Periodontal Status Assessment. One calibrated periodontal specialist performed the periodontal exam. The assessment of intra-examiner calibration was performed through test-retest exercises in ten subjects prior to the initiation of the study. For probing depth (PD), the intra-examiner predictability was 85%, and for clinical attachment loss (CAL) it was 82%. One more calibration

exercise was performed during the study, with the predictability being 83% for PD and 81% for CAL.

The periodontal status was evaluated by measurements of bacterial plaque, bleeding on probing (BOP), periodontal probing depth (PD), and clinical attachment loss (CAL). For the BOP assessment, the gingival tissue was gently dried, the manual periodontal probe (Williams Novatech; Hu-Friedy, Chicago, IL, USA) was inserted in the depth of the gingival sulcus or periodontal pocket, and after 30 s the presence or absence of gingival bleeding was recorded. The oral hygiene was evaluated based on the presence of soft or mineralized deposits on the teeth. PD and CAL were measured at six sites per tooth (mesial-vestibular, central-vestibular, distal-vestibular, mesial-oral, central-oral, and distal-oral) with the same type periodontal probe. The probing depth was considered the distance between the gingival margin and the base of the gingival sulcus/periodontal pocket; CAL was considered the distance between the enamel-cement junction to the base of the sulcus/pocket. Severe periodontitis was acknowledged in subjects with CAL values ≥ 5 mm (not on the same tooth), moderate periodontitis in subjects with CAL values of 3–4 mm (not on the same tooth), and superficial periodontitis in subjects with CAL of 1–2 mm (not on the same tooth) (Newman et al., 2006). The measurements were conducted at baseline (T0) and after 8 weeks (T1).

Treatment Methods. Patients were instructed to follow the nutritional recommendations and continue their usual physical activity throughout the study. All the patients in the study group and those in the control group received non-surgical periodontal debridement that involved ultrasonic scaling (Woodpecker UDS-A-LED, Guilin Woodpecker Medical Instrument Co., Ltd., Guilin, China) and manual root planing (Gracey Standard and Mini curettes—Hu-Friedy, Chicago, IL, USA) (SRP) in one session.

Instructions for dental hygiene were also provided, such as tooth brushing and flossing. Patients were instructed to avoid the use of mouthwash or other antiseptic oral products. In addition to the SRP methods, subjects in the study group received two melatonin tablets (250 mg) containing 3 mg of melatonin, and the control group subjects received two placebo tablets (250 mg) for 8 weeks, 1 h before bedtime. The tablets were taken by direct ingestion with an appropriate quantity of water. There were no visual or tasting differences between the placebo tablets and the melatonin tablets. The potential adverse effects were closely monitored. The subjects who consumed less than 90% of the tablets, either from the study or the control group, were excluded from the study.

Glycated Haemoglobin Measurements.Glycated hemoglobin A1c (HbA1c) was determined for each patient. The method for determining HbA1c was immunoturbidimetric (Boehringer Mannheim, Mannheim, Germany), using a test with a high specificity of anti-HbA1c antibodies considered not to interfere with other forms of hemoglobin.

Statistical Analysis

The data are presented as mean ± standard deviation (SD). The Kolmogorov–Smirnov test was used to assess the data distribution. The independent *t* test was used for the evaluation of the statistical significance between groups at different time points.

1.4.1.3 *Results*

⇒ First study

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 I.1.7. Moreover, 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 6 (T2) and 12 month (T3) assessments ($P<0.001$). 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 (Figure I.1.7.).

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 (Figure I.1.8.).

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. I.1.9.).

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.

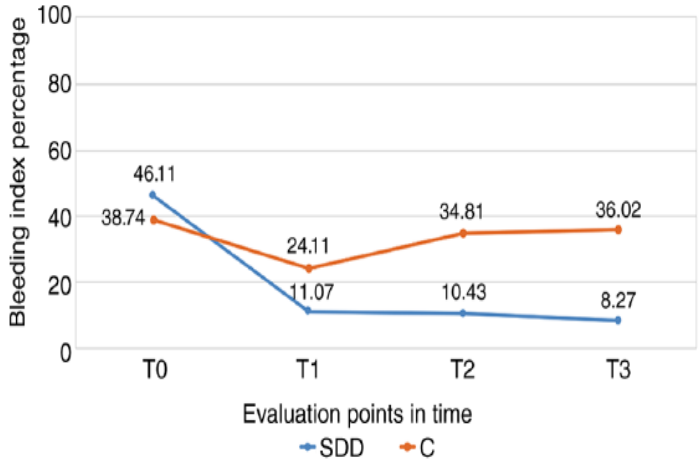


Figure I.1.7. Bleeding index variation at evaluation time points: T0, baseline evaluation (before treatment); T1, evaluation after SDD (3 months from baseline); T2, evaluation at three months after SDD (6 months from baseline); T3, evaluation at nine months after SDD (12 months from baseline). SDD therapy 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.

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 I.1.8.).

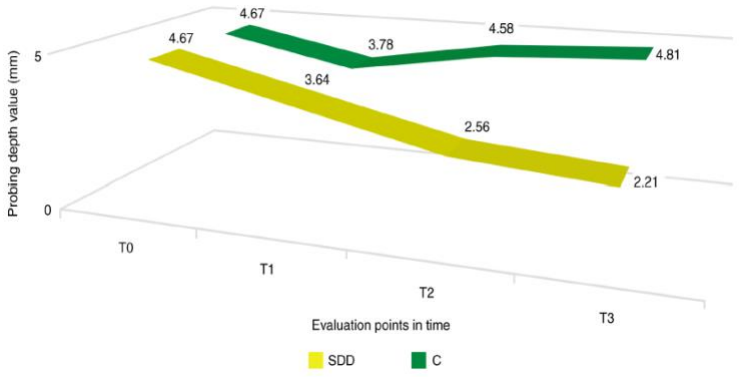


Figure I.1.8. Probing depth variation at the evaluation time points: T0, baseline evaluation (before treatment); T1, evaluation after SDD (3 months from baseline); T2, evaluation at three months after SDD (6 months from baseline); T3, evaluation at nine months after SDD (12 months from baseline). In 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.

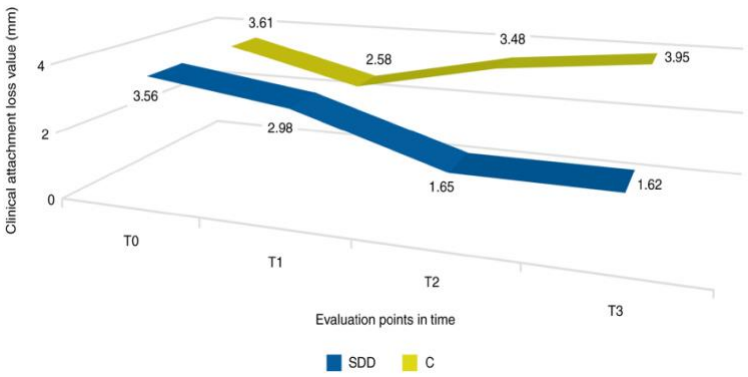


Figure I.1.9. Clinical attachment loss (CAL) variation at evaluation time points: T0, baseline evaluation (before treatment); T1, evaluation after SDD (3 months from baseline); T2, evaluation at three months after SDD (6 months from baseline); T3, evaluation at nine months after SDD (12 months from baseline). 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.

Table I.1.8. Mean values of glycated hemoglobin in the study groups.

	SDD group				**Control group**							
	T0	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**	**V0**	**V1**	**V2**	**V3**
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]

⇒ Second study

All the data from this study had a normal distribution. Fifty-four patients were initially recruited for the study. Four subjects were not able to complete the prescribed drug therapy and were excluded from the study. In total, 50 subjects (study group n = 25; control group = 25) completed the study.

The mean age of the subjects in the intervention and control group was 53.24 ± 3.4 and 52.21 ± 3.1 years, respectively.

No significant differences ($p \geq 0.05$) were observed in terms of demographic characteristics, physical parameters, duration of diabetes, food components, and drugs between the two groups at T0 (Table I.1.9.). No adverse effects of melatonin were observed during the study.

Melatonin therapy significantly decreased the mean values of PD and CAL in the study group after treatment completion ($p < 0.001$); singular SRP therapy also resulted in decreases in the control group but without reaching the level of statistical significance (Table I.1.10.). Both the bacterial plaque index and the gingival bleeding index showed significantly lower values for both study groups at T1, although the decreases were more significant for subjects receiving melatonin therapy.

Table I.1.9. Group characteristics at baseline

Parameter		**Control Group (*n* = 25)**	**Study Group (*n* = 25)**	***p* Value**
Age (years)		52.21 ± 3.1	53.24 ± 3.4	0.4
Gender (%)	Female	40%	44%	0.12
	Male	60%	56%	0.1
Height (cm)		166.20 ± 7.23	168.42 ± 6.99	0.2
WC (cm)		103.31 ± 6.21	102.45 ± 6.45	0.17
HC (cm)		108.22 ± 5.22	107.36 ± 6.08	0.09
WHR		0.95 ± 0.09	0.95 ± 0.07	0.9
Physical activity (minutes)		350.44 ± 120.20	322.62 ± 130.32	0.1

WC = waist circumference; HC = hip circumference; WHR = waist/hip ratio; values are expressed as mean ± standard deviation; $p < 0.05$ was considered statistically significant.

Regarding the periodontitis severity, there were no significant differences between the study groups at baseline. Significant changes were observed for all severity categories (superficial, moderate, and severe) in the study group after 8 weeks, while in the control group we observed a slight decrease in the number of teeth with moderate and severe periodontitis ($p > 0.05$) and a significant increase in the number of teeth with superficial periodontitis ($p < 0.05$) (Table I.1.10.).

At T1, we noticed that conventional scaling and root planing therapy generated improvements in glycemic control, quantified by HbA1c measures, and the differences were significant for the intervention group, which also followed melatonin therapy (Table I.1.10.)

Table I.1.10. Variation in periodontal parameters and glycated hemoglobin values

	Control Group (*n* = 25)		**Study Group (*n* = 25)**		**0**	**1**
	T0	**T1**	**T0**	**T1**		
Periodontal parameters						
PD (mm) (Mean ± SD)	4.53 ± 1.01	4.40 ± 1.02	4.65 ± 1.04	2.27 ± 0.7	.15	0.001
p Value	0.12		<0.001			
CAL (mm) (Mean ± SD)	3.02 ± 0.93	2.98 ± 0.96	3.05 ± 0.56	1.24 ± 0.45	.1	0.001
p Value	0.08		<0.001			
Plaque index (+) (%)	100	48	100	24	.9	.07

	Control Group (*n* = 25)		**Study Group (*n* = 25)**		**0**	**1**
	T0	**T1**	**T0**	**T1**		
p Value	<0.05		<0.001			
BOP (+) (%)	100	40	100	20		
p Value	<0.05		<0.001		.9	.09
Periodontitis severity *						
Superficial	168	191	174	257		
p Value	<0.05		<0.001		.24	0.001
Moderate	257	232	252	202		
p Value	0.08		<0.05		.81	0.05
Severe	72	74	76	53		
p Value	0.72		<0.05		.64	0.05
HbA1c (%)	7.613 7 ± 0.62	7.582 3 ± 0.57	7.624 3 ± 0.71	6.278 1 ± 0.31	.738	0.001
p Value	0.17		<0.001			

T0 = evaluation at baseline; T1 = evaluation after 8 weeks; PD = probing depth; CAL = clinical attachment loss; BOP = bleeding on probing index; HbA1c = Glycated hemoglobin; SD = standard deviation; *p*0 = *p* Value between groups at baseline; *p*1 = *p* Value between groups at 8 weeks; $p < 0.05$ was considered statistically significant. * Severe periodontitis: CAL values ≥ 5 mm, moderate periodontitis: CAL values of 3–4 mm, superficial periodontitis: CAL of 1–2 mm; values expressed as number of teeth.

1.4.1.4 *Discussions*

Diabetes represents a major global concern due to its severe complications, such as vascular, renal, and neurologic pathology, as well as high risk of infections and impaired wound healing that increase the morbidity and mortality in DM patients (Williams 2008). Periodontitis was considered the sixth individual complication of DM due to complex patho-physiological inflammatory interactions (Loe 1993). The presence of periodontitis and, more importantly, of severe periodontal lesions such as alveolar bone destruction leading to tooth loss, in adjunction to a poor response to periodontal classical treatment, is frequent in diabetes subjects. Therefore, setting up an appropriate treatment plan becomes more than a necessity.

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 (Mealey and Oates 2006).

Several studies have shown the positive effects of melatonin and its physiological and pathological implications in the oral cavity (Cutando et al., 2006; Gulle et al., 2014). A study on murine experimental periodontitis model reported that melatonin induced beneficial effects on inflammatory periodontal lesions (Cutando et al., 2006). It was demonstrated that melatonin decreases the number of osteoclasts in DM and periodontitis murine models, improving the alveolar bone loss and periodontal parameters, but it exerted no influence in systemic healthy periodontitis rats (Balci et al., 2016).

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 (PMN) leukocyte 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).

Our study demonstrated favorable changes for all the investigated periodontal parameters at 8 weeks from baseline in patients receiving scaling and root planing alone but statistical significance was achieved only for the plaque index and gingival bleeding. Our results showed that the administration of melatonin for 2 months significantly decreased the mean values of BOP, PD and CAL post-intervention. This is in line with the results of previous studies. Cutando observed that local delivery of melatonin significantly reduced gingival index and PD (Cutando et al., 2015) while, in a previous study, it inhibited the pro-inflammatory markers in DM patients (Cutando et al., 2014).

Similar findings were obtained by a study group led by Bazyar and Javid in an interventional study with systemic supplementation of melatonin (Cutando et al., 2014). Their research included also a potential explanation of the mechanisms involved in the beneficial effects of melatonin in diabetes subjects, with a decrease in inflammatory and oxidative stress markers (Bazyar et al., 2019; Javid et al., 2020).

Therefore, our study supports the possible beneficial effects of melatonin supplementation on the clinical markers of periodontal inflammation and periodontal tissue breakdown in diabetes mellitus patients. Moreover, we observed a significant reduction in the number of severe and moderate periodontitis teeth in subjects following melatonin treatment, in favor of an increased number of superficial periodontitis teeth. Even if the conventional non-surgical periodontal therapy alone followed the same trend, the difference was significant only for superficial periodontitis teeth. Consequently, we can assert that melatonin improves the severity of periodontitis in diabetes patients, bringing additional benefits for these patients.

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 (American diabetes association, 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 (Mealey and Oates 2006). This may be especially true for patients with relatively poorly controlled diabetes and more advanced periodontal destruction prior to treatment.

Periodontal disease represents an infectious-inflammatory disease whose main determinant factor still remains bacterial periodontal pathogens, such as *Porphyromonas gingivalis*, *Treponema denticola*, or *Tannerella forsythia*. The interactions between the bacteria and the host can be modulated by a high diversity of local and systemic risk factors. The general literature data reports no important differences regarding the pathological biofilm between healthy and DM patients. A few heterogenic studies demonstrated a link between the glycemic control and shifts in periodontal biofilm (Polak et al., 2018). Our study showed that both singular SRP and SRP + melatonin intake lowered the microbial plaque, but the decrease was more important when melatonin was added. A small number of studies focused on the potential antibacterial effect of melatonin, such as the one conducted by Srinath (Srinath et al., 2010), but the subject still remains controversial.

The presence of DM does not necessarily represent an absolute indicator for periodontitis but the risk of periodontal disease may be higher in patients with diabetes who have poor glycemic control than in patients with well-controlled diabetes (Takeda et al., 2006). On the other side, a high number of studies demonstrated the favorable effect of non-surgical periodontal therapy (Nishimura et al., 2000). In our study, scaling and root planing alone generated a decrease in terms of HbA1c, but the difference did not reach a statistic threshold, possibly to the short follow-up period.

A prospective study on 2973 systemic healthy subjects investigated the changes in HbA1c across a 5-year period (Demmer et al., 2010). The subjects with severe forms of periodontal tissue breakdown showed a HbA1c value approximately five-fold higher than the periodontal healthy subjects. This was the first study that proved the direct influence of periodontal inflammation on the HbA1c variations. In our study, we also observed a decrease in terms of HbA1c at 8 weeks after scaling and root planing, but the difference was statistically significant only for the subject group who also followed melatonin therapy.

Other studies validated the valuable influence of non-surgical periodontal therapy on improving glycemic control by measures of HbA1c (Grossi 2001; Mealey 2003; Vergnes et al., 2009). The HbA1c reductions found after conventional periodontal therapy are mainly in the range of 0.27–0.48% (Madianos et al., 2018), but there are no sufficient data regarding the maintenance in time of such values. Glycated hemoglobin represents a more reliable indicator for glycemic control than fastening glucose level; high values of glycated hemoglobin are correlated to severe DM complications, and even small reductions in its values mark down the morbidity and mortality from diabetes. It is considered that non-surgical periodontal treatment can exert

such reduced HbA1c values as those obtained by adding a drug to the standard pharmacological regimen (Khaw et al., 2001). Although the recommended levels of HbA1c are <7%, the evidence indicates that there may not be a "safe" threshold for HbA1c. New diabetes treatment strategies are needed to address this growing public health problem.

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 (Mealey and Oates 2006).

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 al., 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., 2014). 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 (American diabetes association 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 (Caton et al., 2000; Walker et al., 2007; 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.

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.

The present study investigated the effects of standard non-surgical periodontal therapy plus adjunctive systemic treatment with melatonin on periodontal parameters and glycemic control (HbA1c) in patients with type 2 diabetes and chronic periodontitis over a period of 8 weeks. Further research is necessary to investigate the benefits of such therapy over a more prolonged period of time both in serum and saliva, as well as the possibility of higher systemic and local benefits of alternative methods of melatonin intake, such as sucking or chewing forms of melatonin tablets.

1.4.1.5 *Final remarks*

The results of our study are in line with the particular direction of the management of complex cases in an integrative manner. The paradigm of "periodontal medicine" is not new, but extensive research is currently performed in order to improve the inter-disciplinary medical approach to patients with systemic conditions and periodontal impairment. Melatonin might represent a highly potent drug in patients with periodontitis, and its beneficial effects could be even of greater importance in patients who present systemic pathologies, such as diabetes mellitus. Moreover, the melatonin regimen proved to be safe, without any adverse effects. We can conclude that a systemic therapy scheme with melatonin might be useful in improving the periodontal status of patients with diabetes and periodontitis by exerting favorable effects not only on local periodontal tissues but also on glycemic control, thus preventing severe complications of both pathologies.

1.4.2. Mitochondrial dysfunction and periodontal disease

1.4.2.1 *Introduction*

Mitochondria have a unique double-membrane structure and represent the central site of metabolism, displaying morphologies, dynamics, and functions specific to each cell and tissue (Friedman and Nunnari 2014). They are responsible for the bulk cellular adenosine triphosphate (ATP) production, through the process of oxidative phosphorylation (OXPHOS). Mitochondria are also the site of important biochemical pathways, including the tricarboxylic acid cycle and a part of the ureagenesis cycle or haem synthesis. They are an important regulator of cell apoptosis and cytoplasmic calcium concentration (Shadel 2008; Nass 1966; Brailoiu et al., 2017).

Mitochondrial deoxyribonucleic acid (mtDNA) is essential for proper mitochondrial function (Shadel 2008). Furthermore, mitochondria are also one of the most important intracellular sites for the formation of reactive oxygen species (ROS). ROS are a family of free radicals that includes superoxide anions, hydroxyl and peroxyl radicals, as well as other compounds capable of generating free radicals (Halliwell 2006). Cells possess numerous systems to counteract the effect of ROS, but their excessive production has been associated with damage at the protein, lipid, and DNA levels (Finkel et al., 2000).

Pathophysiological changes at the mitochondrial level have recently been associated with metabolic pathologies, neurodegenerative diseases, cellular aging, and cancer. The pathological mechanisms underlying these changes are still unknown. In this article, we describe the genetic changes identified at the mitochondrial level in metabolic pathologies and their connection with oxidative stress. Metabolic syndrome (MS) represents the accumu- lation of signs and conditions that, altogether, increase the risk of developing cardiovascular disease, stroke, and type II diabetes mellitus (T2DM). MS is present if at least three of the following five criteria are met: abdominal obesity, high blood pressure, high blood sugar, high serum triglycerides, and low serum high-density lipoprotein (Grundy 2008; Hoffman et al., 2009; Huang et al., 2009). It represents a major health issue in today's society and is significantly connected to socioeconomic difficulties encountered around the world. Early identification, diagnosis, and treatment can improve the long-term life quality and health status of patients with metabolic syndrome (Lancet Diabetes Endocrinol. 2014). The selected and described pathologies (cardiac, obesity, and diabetes) are directly related to oxidative changes generated by excessive ROS and mitochondrial dysfunctions. Therefore, to understand the involvement of mitochondria in these pathologies, we must describe the structure and function of mitochondria, the physiological mechanisms of its biogenesis, and ROS generation at this level.

1.4.2.2 *Mitochondrion Structure and Function*

• **The Electron Transport Chain.** The mitochondrion is a cytoplasmic organelle that consists of double membrane, matrix, and mtDNA. The outer membrane and intermembrane space are relatively per- meable in contrast to the inner membrane which has a restrictive permeability, containing the enzymes required for electron transport)(Sherratt 1991). Mitochondria generate the majority of cellular energy in the form of ATP, through the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH2), and subsequently, the process of oxidative phosphorylation (Chen and Butow 2005). Molecules derived from the catabolism of glucose (glycolysis), fatty acids (beta-oxidation), and amino acids (deamina- tion/transamination) are further referred to the tricarboxylic acid (TCA) cycle to generate the required OXPHOS substrate (Zhao et al., 2019). An electrochemical gradient generated at the level of the inner membrane generates OXPHOS. The electron transport chain (ETC) is made up of five enzyme complexes (I, II, III, IV, and V), located at the level of the mitochondrial inner membrane. Electrons donated by NADH/FADH2 coenzymes are transferred to complex I (NADH: ubiquinone reductase) or complex II (succinate dehydrogenase) of the ETC(Dallner et al., 2000). The two electrons from NADH are given to ubiquinone (UQ)

with the help of cofactors. Subsequently, ubiquinone is reduced to ubiquinol (UQH2). This transfer of electrons triggers the introduction of protons from the matrix into the intermembrane space (through the transfer of two electrons, four protons are introduced). Electrons do- nated by FADH2 are transferred to the UQ via complex II but are not associated with the transport of protons from the matrix into the intermembrane space (Zhao et al., 2019). Afterward, they are transferred to complex III (cytochrome c reductase), made up of cytochromes b and c1. The entire process of electron transfer from UQH2 to cytochrome c is called the Q cycle. Initially, UQH2 binds to complex III, facilitating the access of two protons in the intermembrane space, while two electrons are released, following different paths. The first electron is transferred to cytochrome c1 (at this level it reduces Fe3+ to Fe2+), and from this level, it is then transferred to cytochrome c. The second electron is given to cytochrome b; subsequently, UQ is partially reduced to a molecule called the semi-quinone radical ion (Q-). In the second stage, a new UQH2 molecule is attached to complex III following the same pattern; therefore, a new electron is bound to the cytochrome c level, and the second electron is bound at the Q level with the formation of a UQH2 molecule. At the end of this process, four protons are generated in the intermembrane space. Four electrons are transferred from four cytochrome c molecules to complex IV (cytochrome c oxidase), where molecular oxygen is bound and reduced to water. Finally, at the level of complex IV, eight protons are transferred from the matrix (four are used for the formation of two water molecules, and the other four are transferred to the intermembrane space) (Ahmad et al., 2022). At the end of the electron transport process, using one molecule of NADH, 10 protons are generated towards the intermembrane space In this way, an electrochemical gradient known as mitochondrial membrane potential is produced. Complex V (F0F1 ATP synthase) consists of two domains: extramembrane (F1) and transmembrane (F0). This transport of electrons is associated with the transport of protons from the level of the internal membrane, generating the electrochemical gradient that is necessary for ATP production (Ahmad et al., 2022, Andreyev et al., 2005) (Figure I.1.10.).

• **Mitochondrial DNA Structure.** mtDNA nucleotide sequences were first identified in 1981, and were further re-evaluated and subsequently revised in 1999 (Anderson et al., 1981, Andrews et al., 1999). mtDNA is a double-stranded circular DNA molecule consisting of 16,569 bp which encodes 37 genes, including 13 polypeptides essential for the OXPHOS mechanism, 2 ribosomal RNAs (12S and 16S), and 22 transfer RNAs. mtDNA has a special structure compared to genomic DNA; it does not contain introns, as genes have absent or reduced portions of non-coding bases between them (Protasoni and Zeviani 2021).

Zong et al. described free circulating mtDNA in blood samples with an important prognostic role in various cancers, cardiac arrest, and sepsis (Zong et al., 2016). Subsequently, circulating free mtDNA was identified as a major mediator of innate immunity and systemic inflammatory response. The process of being released into plasma (by an unknown mechanism) results in the activation of neutrophils, mediated by the Toll-like receptor 9 (TLR9) (Shokolenko and Alexeyev 2022). mtDNA is also found in the cytosol. It has been shown that oxidative stress, viral or bacterial infections, or miss-packaging lead to its release and are involved in innate

intracellular immune responses (West et al., 2015). Mitochondrial dysfunctions have been correlated with obesity, diabetes mellitus, and cardiovascular pathologies. An increased amount of glucose is predisposed to the increased production of ROS, with destructive effects at the mitochondrial level (Kowaltowski et al., 2009). The aging process, the reduced action capacity of antioxidants, and the changes produced at the mitochondrial level can be as important causes of metabolic pathologies.

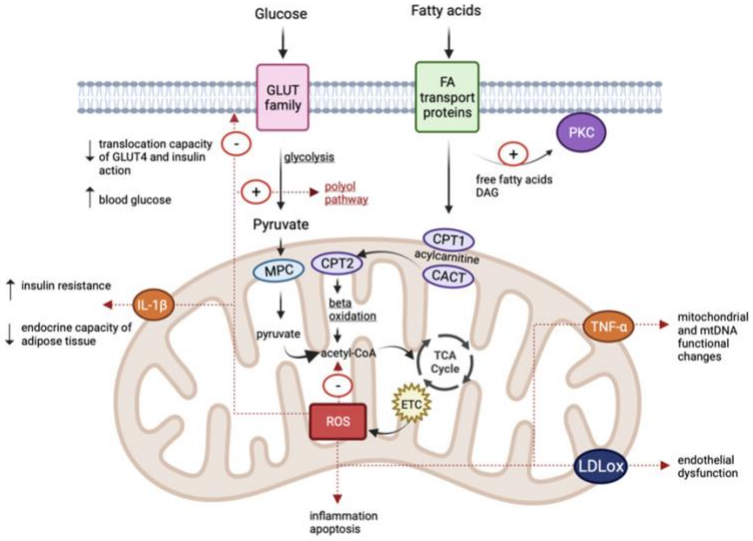


Figure I.1.10. Schematic representation of mitochondrial electron transport chain (ETC). The ETC consists of five enzyme complexes (I, II, III, IV, and V).

• **<u>Mitochondrial Biogenesis and Dynamics</u>.** Most mitochondrial proteins are nuclear-encoded proteins and are translated by cytosolic ribosomes, processed, and imported into the mitochondria via the TIM/TOM system (Yapa et al., 2021). The TOM complex is the translocase of the outer mitochondrial membrane and mediates the importing of nuclear-encoded proteins into the intermembrane space (Araiso et al., 2022). There are two distinct mitochondrial translocase complexes in the inner mitochondrial membrane (TIM) (Tang et al., 2016). The TIM22 and TIM23 complexes recognize and import different classes of proteins (Bauer et al., 2000). Mitochondrial dynamics is essential in maintaining mitochondrial homeostasis and is achieved through two processes: fusion and fission. Imbalances between the two events generate mitochondrial morphological changes, an excess of fission causes the formation of fragmented mitochondria, and an excess of fusion triggers mitochondria elongation.

Mitofusins (Mfn) 1 and 2 are proteins involved in the fusion process of the outer mitochondrial membrane. The fusion of the outer mitochondrial membrane is most often achieved simultaneously with the fusion of the inner membrane, with the latter being mediated by the optic atrophy 1 protein (OPA1). The absence of Mfn cuts off the fusion phenomenon of both membranes. Mitochondrial fission is regulated by dynamin-related protein 1 (Drp1) and fission protein (Fis1) (van der Bliek et al., 2013). Under various metabolic conditions, several

disbalances in such proteins occur during hyperglycaemic conditions, and Drp1 and Fis1 are increased, while Mfn1, Mfn2, and OPA1 are reduced (Kaikini et al., 2017).

Mitochondrial biogenesis is a complex process through which cells increase their mitochondrial mass and require coordination between nuclear and mitochondrial DNA. This process involves mtDNA transcription and translation processes, and the synthesis, import, and association of mitochondrial proteins encoded by nuclear DNA (Popov 2020).

Mitochondrial biogenesis dysfunction has been associated with metabolic disorders such as obesity and T2DM. A decline in the proliferator-activated receptor gamma coactivator-1α (PGC-1α), AMP-activated protein kinase (AMPK), and silent information regulator 1 (SIRT-1) signalling pathways seems to be the underlying mechanism for reduced mitochondrial biogenesis in the diabetic kidney and the diabetic heart as well, with hypoadiponectinemia being reported to impair AMPK-PGC-1α signalling (Popov 2020).

- **Mitophagy.** Autophagy is a natural mechanism which was highly conservated throughout evolution, by which the useless cytoplasmic material is transported to lysosomes for destruction (Galluzzi et al., 2017). Autophagy is influenced by a variety of factors. The autophagic response promotes the adaptation to stress and increases cellular viability (Fuchs et al., 2015). Components of the autophagy response are implicated in regulated cell death (Marino et al., 2014).

The degradation of mitochondria through selective autophagy is referred to as mitophagy, a process that involves the selective sequestration of damaged or dysfunctional mitochondria into double-membraned autophagosomes for later lysosomal destruction. Mitophagy has been described in mammalian cells as being facilitated by two well-studied pathways, ubiquitin-mediated and receptor-mediated, and is essential for maintaining cellular fitness (Ma et al., 2020; Guan et al., 2021).

Mitophagy ubiquitin-mediated pathways are regulated by two key proteins PTEN-induced putative kinase protein 1 (PINK1) and Parkin. Normally, PINK1 is imported into healthy mitochondria via the TIM/TOM system and further degraded by proteolytic reactions. Damaged mitochondria lose membrane potential, which impairs the TIM/TOM system's function, resulting in the accumulation of PINK1 on the outer mitochondrial membrane, which promotes the recruitment of Parkin and the activation of its ubiquitination ligase activity, leading to the ubiquitination of proteins from the outer mitochondrial membrane. Further, Parkin promotes the recruitment of autophagy adaptors, such as optineurin (OPTN) and nuclear dot protein 52 kDa (NDP52), leading to the degradation of damaged mitochondria (Kim et al., 2018; Iorio et al., 2021).

The mitophagy receptor pathway is mediated by receptors embedded in the outer mitochondrial membrane, most notably by NIX (known as BCL2 interacting protein 3 like (BNIP3L)), BCL2 interacting protein 3 (BNIP3), and FUN14 domain containing 1 (FUNDC1), which are characterized by the presence of an LC3-interacting region (LIR) that can directly bind to the autophagy mediator LC3 to promote mitophagy when mitochondria are damaged (Killackey et al., 2020).

Mitophagy is implicated in insulin resistance and some cardiac pathological conditions. The dysfunctional mitophagy mechanism has been linked to the development of insulin resistance (Shan et al., 2022). Moreover, an efficient mitophagical response helps the cardiomyocytes to survive during the nutritional stress in myocardial infarction (Riquelme et al., 2016).

• **Oxidative Stress and Mitochondrial Dysfunctions.** Mitochondrial dysfunctions generated by ROS production in the OXPHOS process are caused by mitochondrial and cellular component damage (DNA, lipids, proteins, and other molecules) (Hu et al., 2011). Metabolic disorders involve the coexistence of numerous risk factors, such as obesity, abnormal cholesterol, and triglyceride values or blood pressure. Oxidative stress is characterized by the imbalance between the production of ROS and the action of antioxidants, with the destructive effect of ROS. Most ROS are generated at the complex I and III levels of the mitochondrial respiratory chain by releasing electrons from NADH and FADH2 to the ETC (Pieczenik and Neustadt 2007).

Free radicals have an increased reactivity due to unpaired electrons, with ROS being one of the most significant recognized classes (Miller et al., 1990; Valko et al., 2007; Sinha et al., 2013). At the mitochondrial level, through the acceptance of an electron by the molecular oxygen, the superoxide anion ($O_2^{\bullet -}$) is generated. It interacts with other molecules or generates secondary ROS (Sinha et al., 2013). $O_2^{\bullet -}$ is subsequently transformed into a more stable compound, hydrogen peroxide (H_2O_2), under the action of the enzyme superoxide dismutase (SOD). The considerable presence of this enzyme at the mitochondrial level supports the importance of $O_2^{\bullet -}$ elimination (Valko et al., 2007; Weisiger and Fridovich 1973; Droge 2002). H_2O_2 can be transported through aquaporins, present at the level of the inner mitochondrial membrane.

Moreover, another possibility of eliminating this compound is diffusion at the cellular level, where it is neutralized and removed with the aid of several antioxidant enzymes such as catalase, glutathione peroxidase, and thioredoxin peroxidase (Holmgren 2000; Nordberg and Arner 2001). When it remains unmetabolized, H_2O_2 interacts with $O_2^{\bullet -}$ and generates the hydroxyl radical ($OH^{\bullet}$), a molecule that is extremely reactive and destructive at the cellular level. Furthermore, in the mitochondrial outer membrane, there is a monoamine oxidase (MAO) enzyme that acts as another source of H_2O_2 (Orrenius et al., 2007), which explains the development of efficient H_2O_2 elimination mechanisms using these organelles (De Grey 2002). Another radical entity is represented by the hydroperoxyl radical ($HOO^{\bullet}$), the protonated form of $O_2^{\bullet -}$, which, however, physiologically develops in small amounts at the cellular level (De Grey 2002). The latter may be involved in lipid peroxidation too.

Another enzyme located in the intermembrane space, p66Shc, has been identified as playing a role in ROS production (Migliaccio et al., 2006). Moreover, singlet oxygen ($^1O_2$) has been identified as playing an important role in the destruction of mtDNA (Cosso et al., 2002). $^1O_2$ is a mitochondrial permeability modulator which can be generated through cytochrome c-catalyzed peroxidation, with carbonyl groups as the substrate (Nantes et al., 1998).Mitochondria are also a source of reactive nitrogen species (RNS). Nitric oxygen ($NO^{\bullet}$) is produced enzymatically by means of nitric oxide synthases (NOS) from amino acids (Radi et al., 2002). L-

arginine is metabolized in the presence of NOS, forming L-citrulline and NO•. NADPH and oxygen are also involved in this reaction (Daenen et al., 2019). Cytochrome c can act as an antioxidant promoting NO• catabolism, but also O2•− to O2 oxidation (Costa et al., 2019). Another antioxidant system is the NAD(P)+-dependent transhydrogenase, located at the inner mitochondrial membrane. It maintains the amount of NADPH in the reduced form by catalyzing the transfer between NADH and NADP+. In addition, mitochondria contain alpha-tocopherol (vitamin E) and UQH2, inhibitors of lipid peroxidation (Costa et al., 2019). (Table I.1.11.). Essential metals such as copper (Cu), manganese (Mn), zinc (Zn), and iron (Fe) are nutrients in various processes that take place at the intracellular level. Copper (Cu) is a cofactor for enzyme function and has an increased redox potential, allowing the transfer of electrons to oxygen and ROS production. An inadequate amount at the cellular level is associated with a compromised immune system, organ dysfunction, and oxidative damage (Barber et al., 2021). Mn is a cofactor for essential enzymes, including catalase and Mn superoxide dismutase (Mn-SOD). Mn-SOD catalyzes O2•− to H2O2 via the Mn2+/Mn3+ cycle, detoxifies free radicals, and prevents oxidative stress. Catalase converts H2O2 to oxygen and water, consequently reducing the production of oxidative stress. Due to these metalloproteins, Mn is involved in antioxidant defence, immune response, and energy production.

Excess Mn has been correlated with oxidative stress and mitochondrial dysfunction. Mn can interfere at the mitochondrial level with oxidative phosphorylation, inhibiting F1-ATPase function and ATP synthesis. Oxidative stress caused by the pro-oxidant capacity of Mn results in increased mitochondrial solubility for protons and ions, a loss of mitochondrial membrane potential, changes in oxidative phosphorylation, and mitochondrial swelling. After chronic exposure to Mn, its accumulation at the mitochondrial level has been observed in neurons and astrocytes (Wolff et al., 2014; Chen et al., 2018; Mezzaroba et al 2019). Increased amounts of Zn suppress the Cu and Fe absorption, causing the production of increased amounts of ROS at the mitochondrial level, disrupting the activity of various enzymes, and activating apoptotic processes. The imbalance of these metals causes structural and functional changes in enzymes, receptors, and transporters (Mezzaroba et al 2019).

Intracellular Fe is found in its reduced form, and it is a cofactor for enzymes located in the cytosol, mitochondria, and nucleus. Catalase, one of the most important antioxidant enzymes, contains four haem groups. Free Fe can exchange electrons with surrounding molecules and form free radicals. Free Fe donates an electron to H2O2 and forms OH• via the Fenton reaction (Menon et al., 2016). Additionally, mitochondria can generate heat due to proton leak. The proton leak results from the activity of fatty acids on uncoupling proteins (UCPs). UCPs belong to the family of mitochondrial anion carrier proteins. Five UCPs have been identified in mammals (UCP1, UCP2, UCP3, UCP4, and UCP5) (Krauss et al., 2005), and they have a purine nucleotide-binding site. ATP, ADP, GTP, and GDP are inhibitors of proton flux and ROS, and fatty acids are activators. UCPs can regulate ion transportation, calcium homeostasis, or synaptic plasticity. UCP1 is expressed in brown adipose tissue, and it is important in the maintenance of body temperature. UCP2-5 have different physiological actions in specific tissues, reducing

oxidative stress. UCP2 is associated with metabolic disorders such as diabetes, obesity, and cardiovascular disease.

Table I.1.11. ROS/RNS and promoters of free radicals; antioxidant systems; and positive and negative effects of oxidative stress on diabetes, obesity, and cardiovascular disease.

ROS/RNS and Promoters of Free Radicals	**Antioxidants System**	**Positive Impacts of Free Radicals**	**Negative Impacts of Free Radicals**	**Ref.**
Superoxide radical anion ($O_2^{\bullet-}$) Hydrogen peroxide (H_2O_2) Monoamine oxidase (MAO) Singlet oxygen($^1O_2$) Hydroxyl radical ($OH^{\bullet}$) Hydroperoxyl radical ($HOO^{\bullet}$) Nitric oxide ($NO^{\bullet}$)	Superoxide dismutase (SOD):Mn-SOD, Cu/Zn-SOD Catalase Glutathione peroxidase Thioredoxin peroxidase NAD/NADP transhydrogenase Cytochrome c oxidase Vitamin E UQH2	Signalling pathways and cell structures synthesis (within fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes) Immune system activity rise Induction of mitogenic response Vasodilation Angiogenesis Wound healing	Lipid peroxidation Damage to cell membranes and lipoproteins Cytotoxic and mutagenic compounds Conformational modifications of proteins DNA lesions Loss of epigenetic information Hypertension Atherosclerosis	(Pizzino et al., 2017, Jitca et al., 2022)
Disease	**Biomarkers**	**Mechanism of action and effects of oxidative stress**		
Diabetes	↑malondialdehyde ↑8-isoprostane ↑4-hydroxynonenal ↑glycated haemoglobin ↑advanced oxidation protein products ↑protein carbonyls ↓glutathione ↓superoxide dismutase ↓catalase	Lipid peroxidation Protein oxidation Decreased insulin activity Hyperglycaemia Stimulation of the polyol pathway Stimulation of glucose autoxidation Increase in advanced glycosylation end products mtDNA and proteins conformational modifications		(Tiwari et al., 2013., Newsholme et al., 2016, Ding et al., 2021, Leguisamo et al., 2012)
Obesity	↑tumour necrosis factor-α ↑nuclear factor-κB ↑interleukin-1β ↑interleukin 6 ↑plasminogen activator inhibitor 1 ↓superoxide dismutase ↓catalase ↓vitamin A ↓vitamin E ↓vitamin C	Excess of pro-inflammatory cytokines and expression of adhesion molecules and growth factors Depleted antioxidant levels Increase in free fatty acids Thrombosis and insulin resistance		(Marseglia et al., 2014)
Cardiovascular Disease	↑oxidized low-density lipoprotein ↑tumour necrosis factor-α ↑nuclear factor-κB ↑interleukin-1β ↑interleukin 6 ↑8-Hydroxyl-2′-	Endothelial dysfunction Inflammation in blood vessels Atherosclerosis Hypertension Cardiac hypertrophy Cardiomyocytes apoptosis Oxidative damage in DNA		(Stephens et al., 2006, Kono et al., 2006, Vita et al., 1998, Polidori et al., 2004, Blankenberg et

ROS/RNS and Promoters of Free Radicals	**Antioxidants System**	**Positive Impacts of Free Radicals**	**Negative Impacts of Free Radicals**	**Ref.**
	deoxyguanosine ↑myeloperoxidase ↑F2-isoprostanes ↑biopyrrins ↓vitamin C ↓glutathione peroxidase 1 ↓total antioxidant status	Lipid peroxidation		al.,2003, Ndrepepa et al., 2008, Lahera et al.,2017, Chistiakov et al., 2018)

Note: ↑ is increase, ↓ is decrease.

1.4.2.3 *Insulin resistance and mitochondrial Dysfunctions*

Type 2 diabetes mellitus (T2DM) is a chronic pathology that requires continuous medical care through pharmacological treatment, but also a reduction in risk factors involved in the etiopathogenesis of the disease. Currently, this condition is characterized by a permanent increase in incidence and prevalence (Melmer and Laimer 2016). In recent years, we notice an increase in interest towards research and the identification of mitochondrial changes and their involvement in chronic diseases. There are numerous studies which confirm that excess ROS and the presence of mitochondrial dysfunctions contribute to the development of metabolic pathologies and insulin resistance. Obesity, diabetes, and cardiovascular disease have been linked to mitochondrial dysfunction (Turner et al., 2008; Korkmaz et al., 2013; Chen et al., 2014; Das et al., 2016; Yan et al., 2019).

Hyperglycaemia and T2DM are directly linked to mitochondrial activity, function, and oxidative stress. Mitochondria produce the largest amount of ROS and ATP. With regards to hyperglycaemic status, the amount of ROS increases and triggers changes in cellular homeostasis with the generation of lesions at this level (Szendroedi et al., 2011). The increased production of $O_2^{\bullet -}$ affects the translocation capacity of glucose transporter 4 (GLUT4) from the intracellular level to the plasma membrane, resulting in a decrease in insulin action at the tissue level and an increase in blood glucose amount. Hyperglycaemia generates an excessive production of ROS which favours the appearance of mitochondrial changes and stimulates the polyol pathway, glucose autoxidation, and an increase in advanced glycosylation end products in diabetic patients (Wu et al., 2004). Changes at the OXPHOS level, a reduction in NADH oxidoreductase, and citrate synthase activity induce insulin resistance (Ahmad et al., 2017). Fatty acid catabolism, a mechanism called beta-oxidation, is also carried out at the mitochondrial level. The reduction in fatty acid oxidation, together with the accumulation of lipids and diacylglycerol, drive both the activation of protein kinase C and increased ROS production. Thus, there are changes in the mitochondrial functioning mechanism that affect ATP synthesis. A compromise of mitochondrial function causes a lipid excess and the development of insulin resistance(Gastaldi et al., 2008). The amplification of oxidative stress generates and maintains inflammation, causes lipid peroxidation, and initiates changes in the insulin signalling mechanism. Insulin resistance generated by hyperlipidaemia induces changes in mtDNA and proteins.

In diabetic patients, at the level of mononuclear cells, increased amounts of ROS were identified, as well as spherical and hyperpolarized mitochondria, thus indicating dysfunctions at

this level (Rendra et al., 2019). The effect of ROS was also proven in pancreatic β cells; the changes including volume and shape modifications, as well as changes in mitochondrial function. They affect ATP-dependent K+ channels and insulin secretion. This aspect can also be explained by the lower amount of antioxidants in β-cells(Darenskaya et al., 2021) . The reduction in ATP production and the increase in ROS at the muscle level can trigger an increase in insulin resistance and diabetes (Short et al., 2005) (Figure I.1.11).

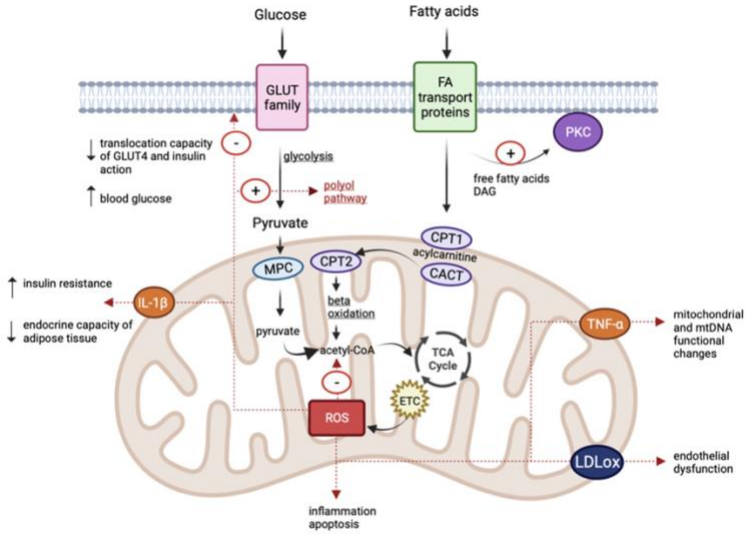


Figure I.1.11. Schematic representation of common pathophysiological mechanisms in diabetes, obesity, and cardiovascular disease. (TCA, tricarboxylic acid cycle; ETC, electron transport chain; CPT1, carnitine palmitoyl-transferase 1; CPT2, carnitine palmitoyl-transferase 2; CACT, carnitine-acylcarnitine translocase; MPC, mitochondrial pyruvate carrier; IL-1β, interleukin IL-1β; TNF-α, tumour necrosis factor-α; LDLox, oxidized LDL; DAG, diacylglycerol).

Changes in mtDNA and genomic DNA have also been identified in patients with diabetes mellitus. A new m.8561C>G mutation in MT-ATP6/8 was identified and correlated with T2DM and hypergonadotropic hypogonadism (Rovira-Llopis et al., 2017). The m.3242A>G mutation and the 10.4-kb deletion were associated with diabetes and deafness. It is considered that mitochondrial mutations can accumulate over time (an aspect that correlates with the aging process and neurodegeneration). There is also a correlation between mitochondrial epigenetic changes and insulin resistance (Zheng et al., 2015). These changes at the mtDNA level suggest the importance of continuing research in this field. Changes in the COX7A1 and NDUFB6 genes have been identified in people with insulin resistance and T2DM. Although they are nuclear genes, they encode subunits of OXPHOS complexes I and IV, respectively (Ling et al., 2007).

1.4.2.4 *Obesity and mitochondrial dysfunctions*

Obesity is associated with changes in carbohydrate and lipid metabolism, but also with insulin resistance, an increased risk of cardiovascular pathologies, and diabetes mellitus (Cheng et al., 2014). It represents one of the components of the metabolic syndrome and a cause of the

development of numerous chronic conditions. A caloric imbalance causes the hyperplasia and hypertrophy of adipose cells. Adipose tissue secretes adipokines that have immunoregulatory properties (De Mello et al., 2018). The maintenance of the inflammatory process by an increase in leptin and resistin (pro-inflammatory factors) and decrease in adiponectin (an anti-inflammatory factor) triggers an increase in ROS and oxidative stress (Prasun 2020). Mitochondrial dysfunctions cause interleukin IL-1β secretion, which affects peripheral insulin sensitivity and interferes with the endocrine capacity of the adipose tissue (Xu et al., 2022). In hyperlipidaemic diets, mitochondrial fatty acid oxidation increases, inducing the subsequent generation of increased levels of acetyl coenzyme A, which further amplifies the levels of NADH and FADH2 in the tricarboxylic acid cycle, as well as the accumulation of acylcarnitine and ROS formation. The excess of free fatty acids at the adipocyte level activates NADPH oxidase enzymes with an increase in ROS. Oxidative stress causes inflammation and boosts lipid peroxidation, disrupting insulin's mechanisms of action (Cade 2018) (Figure 2).

Cells can release extracellular vesicles containing mitochondria. Recipient cells receive mitochondria through an extracellular vesicle–cell fusion event (Boudreau et al., 2014). The intercellular transfer of mitochondria has been implicated in many pathological conditions such as stroke, pulmonary hypertension, and obesity (Hayakawa et al., 2016).

Adipocytes transfer mitochondria to macrophages in adipose tissue, generating a new population of macrophages, which is greatly diminished in patients with obesity due to increased lipid intake because of reduced mitochondrial uptake by macrophages. Mitochondrial uptake is mediated by heparan sulphate and it manifests low levels in obese subjects. The exostosin (EXT) 1 gene and the EXT2 heterodimer are also associated with the maintenance of lipid metabolism homeostasis and glucose levels. The presence of deletions in the EXT1 gene in myeloid cells reduces heparan sulphate levels, decreases mitochondrial transfer, and increases adipose tissue accumulation (Brestoff et al., 2021).

It has been shown that numerous components of the ETC have decreased expression in visceral adipose tissue in women with diabetes. A decrease in the expression of OXPHOS genes was also noted in these patients (Pinti et al., 2019). A reduction in the number of mtDNA copies at blood, muscle, and adipose tissue level has been reported in obese subjects and those with type 2 diabetes mellitus (Kaaman et al., 2007; Lee et al., 2014). An association between the reduction in mtDNA copy numbers and increased mtDNA methylation in the D-loop region was identified in obese individuals .

1.4.2.5 *Cardiovascular disease and mitochondrial dysfunctions*

Cardiovascular disease is the leading cause of death worldwide. There are a large number of factors involved in the development of cardiovascular pathologies. Mitochondrial changes produced during the aging process explain the functional deficit encountered in cardiovascular pathologies (Cox et al., 2022).

Cardiomyocytes contain numerous mitochondria to generate a large amount of ATP (Zinovkin et al., 2019). At the cardiac level, mitochondria are located in interfibrillar, subsarcolemmal, and perinuclear regions (Vásquez-Trincado et al., 2016). Mitochondrial

structural changes occur in cardiac pathologies, with the formation of megamitochondria (giant mitochondria generated by fusion). Shape changes are also encountered (cristae reorientation and the presence of intramitochondrial cylinders).

The largest amount of ATP is obtained at the cardiac level via the beta-oxidation of fatty acids but, depending on availability, glucose can be used as an energy source. In cardiac pathologies, insulin signalling is altered, affecting metabolic flexibility. Therefore, the amount of ATP decreases.

Oxidative stress and mtDNA changes are identified in patients with cardiovascular pathologies. At the cardiac level, ROS are generated through complexes I and III in neutrophils, endothelial cells, and myocytes. The increase in ROS causes endothelial dysfunction, inflammation in blood vessels, and the generation of oxidized LDL at the arterial level. Ultimately, these changes trigger atherosclerosis, hypertension, and cardiac hypertrophy. In addition, mitochondrial dysfunctions stimulate enzyme activation and induce cardiomyocyte apoptosis. It has been found that ROS association, together with the increase in pro-inflammatory factors such as tumour necrosis factor-α (TNF-α), causes mitochondrial and mtDNA functional changes, favouring the development and progression of cardiovascular pathologies (Suematsu et al., 2003).

Several studies indicate methylation changes in cardiovascular pathologies. Extensive methylations were found in the MT-CO1, MT-CO2, MT-CO3, and MT-TL1 genes in subjects with these conditions, with the methylation degree of these genes being a potential predictive marker of cardiovascular pathologies in obese patients (Stoccoro and Coppede 2021).

Pharmacological Strategies and Lifestyle Interventions in Mitochondrial Dysfunctions

Mitochondrial dysfunctions are involved in the pathophysiological mechanisms that generate metabolic disorders. In this context, interest shown toward mitochondria can generate new optimal therapies for these pathologies. The aim is to slow the progression of mitochondrial dysfunctions and decrease ROS with a subsequent reduction in oxidative stress.

Recent studies support the idea that physical activity improves insulin sensitivity and mitochondrial function in muscle tissue in patients with T2DM. Caloric restriction reduces excessive ROS production and inhibits inflammation, thus facilitating the prevention and treatment of metabolic pathologies (Rocki et al., 2008; Holloway 2009; Joseph et al., 2014). In recent years, it has been suggested that a diet rich in polyphenols might be beneficial in subjects affected by metabolic dysfunction (Jabczyk et al., 2021).

Due to the multitude of discoveries in the field, interest in mitochondrion-directed therapy has increased. Although numerous action mechanisms are known, the challenge is represented by the transportation of active substances at this level due to the barriers that precede mitochondrial localization. In this regard, some of the drugs that may represent a new therapeutic solution in metabolic pathologies are selected further.

ETC components may represent a target in pharmaceutical interventions. Rotenone and Annonaceous acetogenins inhibit NADH ubiquinone oxidoreductase. Moreover, metformin is a complex I inhibitor, a drug used in diabetes that increases glucose consumption. Vitamin E

analogues (α-tocopheryl succinate) and 3-bromopyruvate act on complex II. Complex III inhibitors are antimycin A and myxothiazole. Cyanide is a complex IV inhibitor. There are numerous pharmaceutical agents that act on complex V (oligomycin, apoptolidins, resveratrol, dindolyl methane, and aurovertin) (Toogood 2008; Milane et al., 2018).

A subfamily of mitochondrial proteins is represented by uncoupling proteins (UCPs). UCPs facilitate H+ transport, ultimately generating heat. Fatty acids stimulate UCP transport. In addition, it was highlighted that adrenaline and prostaglandins determine the upregulation of UCPs. They are involved in common pathologies, with UCP1 and UCP2 being associated with diabetes and obesity. Carbonyl cyanide m-chlorophenyl hydrazone and 2,4-dinitrophenol are uncoupling agents for oxidative phosphorylation. UCPs represent a good pharmacological target for treating obesity and diabetes (Ishigaki et al., 2005; Nubel and Ricquier 2006).

From a pharmacological point of view, sirtuins (silent information regulator proteins) are involved in the regulation of glucose and lipid metabolism at the cellular level (Turkmen et al., 2014; Huynh et al., 2013; kitada et al., 2013). Resveratrol activates SIRT 1, and improves insulin resistance through its antioxidant properties (Lagouge et al., 2006).

Mitochondrial fission inhibitors such as mitochondrial division inhibitor 1 (Mdivi 1), P110, and dynasore have been identified as playing a role in ameliorating oxidative stress 9Meuer et al., 2007, Cassidy-Stone et al., 2008, Qi et al., 2013). Mdivi 1 inhibits the GTPase activity of DRP1, improves myocardial infarction after ischemia, and restores mitochondrial changes caused by excess ROS. It was demonstrated in a study conducted on an animal model that Mdivi 1 reduces inflammation and oxidative stress and improves endothelial function. Dynasore inhibits the mitochondrial fission phenomenon and increases the survival chances of cardiomyocytes after ischemia. P110 is a peptide that inhibits DRP1 activity, with a beneficial effect on mitochondrial morphology and function. Its effect is demonstrated in cancer and neurological pathologies, but in metabolic dysfunctions, it is still being studied. Cyclophilin D is a mitochondrial protein known to regulate the mitochondrial permeability transition pore (Amanakis et al., 2020). This can represent a therapeutic target.

Antioxidants

Several studies have highlighted the beneficial effect of antioxidant use in metabolic pathologies (He et al., 2014; Mailloux et al., 2016; Silva et al., 2016; Ni et al., 2016;). An increase in antioxidants, such as vitamin E, vitamin C, coenzyme Q, and N-acetylcysteine (NAC), is indicated for their oxidative stress reduction (Mehta et al., 2002; Yorek et al., 2003). NAC is the acetylated form of the amino acid L-cysteine and is a source of the thiol group (SH) with the potential to stimulate glutathione biosynthesis. It inhibits the activity of p38 MAP kinase, nuclear factor kappa B, and redox-sensitive activating protein-1; increases SH availability; and interacts with NO• forming nitrosothiols. NAC inhibits the oxidative degradation of NO•, thus decreasing the amount of nitrogen dioxide, peroxynitrite, and nitrotyrosine. NAC therapy has beneficial effects on oxidative stress, inflammation, epithelial dysfunction, and hypertension (Rani et al., 2020). Coenzyme Q10 (CoQ10) is an antioxidant and plays an important role in lipid structures (cell membranes and lipoproteins). It is localized at the

mitochondrial and extramitochondrial levels and has three oxidation states (oxidized, partially reduced, and fully reduced). Combining oxidized CoQ10 with selenium improves the protection against cardiovascular pathologies. Patients with T2DM have low amounts of COQ10, and supplementation with CoQ10 and selenium leads to the reduced formation of advanced glycosylation end products. Moreover, the use of CoQ10, together with selenium and vitamin C, has synergistic antioxidant effects (Aaseth et al., 2021).

Although the beneficial effect of using conventional antioxidants in metabolic pathologies is known, in some clinical trials, the results of their benefits are contradictory. Salehi et al. highlighted the adverse effects of inappropriately using nontargeted antioxidants (vitamin A, vitamin C, vitamin E, and β-carotene). Excessive vitamin A intake (more than 10,000 IU) has been associated with increased teratogenicity risk or birth defects. Vitamin C can be metabolized to oxalate, increasing the risk of calcium oxalate kidney stones, and vitamin E may increase prostate cancer risk. The excessive use of antioxidants can decrease ROS production and stimulate the compensatory upregulation of mitogen-activated protein kinase (MAPK) pathways. Furthermore, it is difficult to calculate the dose of nontargeted antioxidants used for disease treatment because there are many uncertainties about the mechanism of absorption and how they are metabolized in different organs. Due to the adverse effects of conventional antioxidants at the cellular level, the production and use of mitochondria-targeted antioxidants are becoming more relevant (salehi et al., 2018; Jiang et al., 2020).

Mitoquinone (MitoQ), mitovitamin E (MitoE), and MitoTEMPO are mitochondria-targeted antioxidants and are synthesized by attaching the antioxidant to the lipophilic triphenylphosphonium cation (TPP+). TPP+ is the most widely used lipophilic cation. To permeate the mitochondrial membrane, the substances must permit the optimal lipophilic level. If the molecule has low lipophilicity, it does not penetrate the mitochondrial membrane, and if lipophilicity is increased, it accumulates at the membrane level. Due to the processes carried out in the ETC, the increase in the mitochondrial membrane potential allows the passage of these modified antioxidants inside the mitochondria and the action at this level. MitoQ is an antioxidant in which TPP+ is bound to the UQ. It accumulates at the mitochondrial membrane, decreases the production of ROS, and prevents the potential changes caused by oxidative stress (Kelso et al., 2001; Yang et al., 2021). MitoQ is now described as a potential pharmaceutical compound in neurodegenerative pathologies (Mao et al, 2013; Powell et al., 2015; Yin et al., 2016). MitoE is a therapeutic agent that crosses the mitochondrial membrane and protects mitochondria against excess ROS (Smith et al., 1999). SkQ1 is also a mitochondrial antioxidant formed from the conjugation of TPP+ with plastoquinone (Titova et al., 2018). SkQ1 has antioxidant effects at low concentrations and can bind to cardiolipin, preventing its oxidation. MitoQ and SkQ1 can reduce oxidative stress, decrease protein oxidation, and prevent lipid peroxidation and cell apoptosis. Animal model studies have revealed their benefits in metabolic syndrome, obesity, and ischemia–reperfusion injury. Mitochondrial toxicity is the limiting factor in the use of TPP+ antioxidants. This requires proper dosing and concentrations below levels at which mitochondrial membrane damage occurs.

Liposomes are bilipid membrane vesicles used to transport certain bioactive substances. Liposome-encapsulated antioxidants are composed of cholesterol, phosphatidylcholine, phosphatidylglycerol, cholesterol, and antioxidants (quercetin, NAC, and vitamin E). Liposome-encapsulated antioxidants penetrate at the cellular level through the phenomenon of pinocytosis, the liposomal components fuse with the mitochondrial membrane, and the antioxidant is released at the mitochondrial level. MITO-Porter is a novel system used for transporting bioactive components to the mitochondrial level.

It represents a liposomal nanocarrier made up of 1,2-dioleoyl-sn-glycerol-3-phosphatidylethanolamine, sphingomyelin, and stearylated octa arginine peptide (R8). MITO-Porter binds at the mitochondrial level due to the interaction between R8 and negatively charged mitochondria (Yamada et al., 2017).

1.4.2.6 *Final remarks*

Mitochondria are versatile organelles, responsible for most of the cellular chemical energy production. The structure and function of this subcellular organelle are peculiar, due to the abounding molecular mechanisms that take place at this level. They are responsible for cell survival, apoptosis, and homeostasis. These benefits coexist with the high degree of errors that can occur at the mitochondrial level through ROS. Mitochondrial changes have been identified in metabolic dysfunctions including insulin resistance/diabetes, hypertension, and dyslipidaemia, but also in cancer or neurodegenerative pathologies. Recent studies have identified changes in mitochondrial biogenesis and dynamics, as well as the presence of oxidative stress in metabolic dysfunctions. In diabetic patients, at the level of mononuclear cells, spherical and hyperpolarized mitochondria were identified, indicating dysfunctions at this level. In pancreatic β cells, changes including volume, shape modifications, and mitochondrial dysfunction have been reported as well.

The reduction in ATP production and the increase in ROS at the muscle level can trigger an increase in insulin resistance and diabetes. In obesity patients, the excess of free fatty acids at the adipocyte level activates NADPH oxidase enzymes, with an increase in ROS production. Oxidative stress causes inflammation and boosts lipid peroxidation, disrupting insulin's mechanisms of action. The increase in ROS causes endothelial dysfunction, inflammation in blood vessels, and the generation of oxidized LDL at the arterial level in cardiovascular diseases. To reduce the progression of metabolic pathologies, lifestyle interventions, physical exercises, and dietary changes are indicated. A balanced diet, including fruits, legumes, and vegetables of different colours, is recommended.

Moreover, new pharmaceutical strategies that can improve the prognosis of these conditions are being investigated and developed. Several studies have highlighted the beneficial effect of using antioxidants in metabolic pathologies; vitamin E, vitamin C, coenzyme Q, and NAC administration are indicated for their oxidative stress reduction effect. However, in some clinical trials, the results of their benefits are contradictory. Due to the adverse effects of conventional antioxidants at the cellular level, the production and use of mitochondria-targeted antioxidants are becoming more relevant. The epigenetic role in this context is not fully

understood, but changes in mtDNA have been identified which could be meaningful in the identification and design of the next therapeutic strategies. It is considered that mitochondrial mutations can accumulate over time.

Several studies indicate methylation changes in cardiovascular pathologies, and the methylation degree of specific genes can be a potential predictive marker of cardiovascular pathologies in obese patients. Difficulties in this field arise due to the particularities encountered at the level of each individual. Large and complex studies are needed in order to identify and detail the changes at the mitochondrial level and the therapeutic approaches.

1.4.3. Immunity status and periodontal disease

1.4.3.1 *Introduction*

Periodontal disease is a frequent oral disease of bacterial cause which comprises of gingivitis and periodontitis. In this pathology, the tissues that surround and support teeth are affected to various degrees according to severity (Germen et al., 2021).

Gingivitis is the most frequent form, and it is expressed through edema, bleeding and pain, and if left untreated it develops into periodontitis in which periodontal attachment and supporting bone is lost gradually (Barret 1984; Brito et al., 2022).

Periodontitis is a highly prevalent disease that affects approximately 50% of adults in its mildest forms, this percentage being higher in subjects over 65 years of age. Severe periodontitis is the sixth most common human disease and it affects nearly 12% of the global adult population (Eke et al., 2020).

The severe form of the disease is characterized by major loss of periodontal tissues, both superficial and profound, which leads to tooth loss if left untreated, this in turn leads to an affected nutrition, speech impediments, low self-esteem, and an overall diminished quality of life (Stetiu et al., 2010).

All things considered, severe periodontal disease constitutes an important social, healthcare and economic strain, and is at the crux but also an outcome of social disparity worldwide.

Moreover, in the near future, the prevalence of periodontitis is likely to increase globally because of an aging population and therefore an elevated preservation of teeth (Botelho et al., 2022).

Another major issue is the association of periodontal disease with other common systemic conditions such as cardiovascular disease, adverse pregnancy outcomes, diabetes, kidney disease, rheumatoid arthritis, Alzheimer’s disease, chronic obstructive pulmonary disease, and cancer (Genco and Sanz 2020).

Microorganisms and their products, which form the oral biofilm, together with inflammatory mediators, disseminate from periodontal tissues via blood vessels in the entire body, thus accounting for the link between periodontitis and other systemic diseases and conditions (Bui et al., 2019).

Recently, major advances have been made in the etiopathogenesis of periodontal disease, in the recognition and description of the significant risk factors that increase the risk of developing
periodontal diseases, and in the increasing proof of the epidemiologic and mechanistic associations between systemic diseases and periodontitis.

Even though systemic inflammation, diabetes, cardiovascular diseases and adverse pregnancy outcomes are still the focal point of research regarding these correlations, nowadays other systemic diseases, such as rheumatoid arthritis, obesity and metabolic disease, respiratory diseases, cancer, and neurodegenerative diseases, have been linked with periodontal disease (Hirschfeld et al., 2021).

Leukemia is a blood disease of a malignant nature that is defined as a disorganized proliferation of red and white and blood cells in the bone marrow, resulting in undifferentiated cells (called blasts) that lose normal cell functionality (Gundesen et al., 2019).

These undifferentiated cells, in time, are able to infiltrate other tissues and organs, including the oral cavity. The infiltration of the tissue, along with blood modifications, can significantly alter the oral environment, for example causing edema and gingival bleeding, which in most patients are the initial signs and symptoms of the disease (Chowdhri et al., 2018; Quispe et al., 2022).

Moreover, leukemia patients are treated with high doses of chemotherapy and/or radiotherapy, which have various effects on the oral cavity and on periodontal tissues. Another issue is the reduced capacity of hospitalized patients to maintain proper oral hygiene during systemic disease treatment (Angst et al. 2020).

Chemotherapy is a widely used treatment for a variety of cancer forms (Chabner et al., 2005). Its therapeu- tic action consists of damaging cancer cells, but also healthy cells that have a high turn-over rate, thus leading to side effects (American Cancer Society, 2012). The majority of patients undergoing chemotherapy, up to 86%, have reported side effects during treatment and have shown cumulative toxic effects (Pearce et al., 2017). The most cited prevalent adverse effects include a variety of immediate and late signs of toxicity, such as fatigue, nausea, alopecia, immunosuppression, insomnia, gastric discomfort (Sun et al., 2005; Vardy et al., 2006), drug resistance, and infertility [6]. Oral cavity side effects include mucositis, which is among the most prevalent, bacterial, fungal and viral infections, neuro- logical alterations, dysgeusia, dental alterations, hyposialia and xerostomia, tendency for hemorrhage and osteonecrosis (Epstein et al., 2012).

Platinum-based drugs, such as cisplatin and oxaliplatin, are often used in the treatment of malignancies due to their effectiveness, although they frequently induce severe, dose- limiting side effects, such as nephrotoxicity and neurotoxicity (Oun et al., 2018). On the other hand gemcitabine, a potent and specific deoxycytidine analog, is relatively well tolerated and in fewer cases does it induce side effects, such as anemia, neutropenia, or neutropenic fever (Toschi et al., 2005). In plasma, cisplatin can be found in two forms: protein bound and free circulating, which represents the active form of the drug. Up to 90% of the administered cisplatin dose becomes

bound and inactivated by plasmatic proteins (Gift et al., 2004). Similar to cisplatin, oxaliplatin undergoes a series of biological transformations once it reaches the bloodstream and also splits into three fractions: total platinum, erythrocyte platinum, which is the protein bound form, and free oxaliplatin (Wang et al., 2007). Gemcitabine is one of the most used drugs in oncology, ranking third worldwide. It represents the base treatment for pancreatic cancer (Vermorken et al., 1984) and a series of solid tumors, such as breast, ovary, pulmonary, and urinary bladder cancer (Bonetti et al., 1995; Levi et al., 2000; Urien and Lokiec 2004).

Saliva is valuable fluid from a diagnostic standpoint, which is used in several circumstances due to its complex and varied composition, which is often tightly related to the general status. Is has been used several times previously as an alternative to blood testing, for DNA analysis (Solomon et al., 2015) or quantitative and qualitative testing of various drugs. Due to these reasons and the non-invasive character of the determinations, especially for oncology patients, we have chosen to use salivary analysis in the present study.

Determining chemotherapy drug levels in saliva through spectrometry may offer useful information without additional risks (Burris et al., 1997). Collecting saliva samples, however, can prove difficult due to hyposialia and xerostomia that may often occur in chemotherapy patients and it is a possible reason for the limited number of studies in the scientific literature on this subject.

The aim of this study was to evaluate the concentration of chemotherapy drugs administered systemically in saliva and analyze the oral and periodontal clinical modifications in correlation with the chemotherapy levels present in the salivary fluid.

1.4.3.2 *Materials and Methods*

The study was conducted on 29 patients admitted to the Oncology Clinic of Hospital Victoria in Iasi between October 2018–May 2019, aged from 43 to 80 years old.

Inclusion criteria for the present study: patients suffering of systemic cancer and currently undergoing chemotherapy. The exclusion criteria were: non-cancer patients, patients with infectious or inflammatory diseases affect the periodontal status (with the exception of systemic cancer), patients that have received periodontal treatment in the last 6 months or antibiotic/anti-inflammatory treatment in the past 3 months, pregnant patients or minors.

The total number of patients were split into three groups: patients receiving cisplatin (N = 5), oxaliplatin group (N = 18) and gemcitabine group (N = 6). All the collected data was compared between the three groups.

- General and Periodontal Data Collection

The data collection procedure was performed by gathering the general information of patients, identification, data regarding the oncology diagnostic (localization, stage), data about the chemotherapy treatment (administered drug, reported side effects) and clinical periodontal data on Ramfjord teeth (16, 21, 24, 36, 41 and 44): Sillness and Loe gingival index (GI), which provides a periodontal overview of the clinical aspect and bleeding of gingival tissues, gingival bleeding index (GBI), which further quantifies the amount of bleeding in regards to the presence of inflammation, CPITN index which offers information of periodontal damage, as well as

treatment needs, probing depth (PD) and clinical attachment loss (CAL), which offers an in-depth view of present periodontal damage.

The present study was approved by the Ethics Committee of 'Grigore T. Popa' University of Medicine and Pharmacy (Iasi, Romania). All patients were informed in regards to the procedures specific to the present study and signed written consent for participation.

• Saliva Sample Collection and Analysis through Mass Spectrometry and Fluid Chromatography

The collection of saliva samples was performed on patients included in the study before the clinical examination and after a light rinse with water of the oral cavity. The saliva was collected in sterile recipients until at least 5 mL of total saliva was gathered. The collecting procedure was performed before the start of the current chemotherapy iv (T0), repeated at 30 min after chemotherapy administration was concluded (T1) and at 2 h (T2), obtaining a total of 3 samples per patient; each sample received was appro- priately inscribed with the patient initials, time of collection and type of chemotherapy administered. It is to be noted that T0 indicates the end of the previous chemotherapy administration cycle. T1 was chosen as a collection time due to the fact that maximum concentrations of chemotherapy agents seem to be detected 30 min after administration according to the literature (McCarthy et al., 1998). T2 reports the rate of decrease in the above-mentioned types of drug concentrations. All concentration values were comparatively evaluated in order to determine the pharmacodynamic curve of each chemotherapy agent included in this study.

The three chemotherapy agents used in this study were cisplatin (CIS), oxaliplatin (OXA) and gemcitabine (GEM) provided by the European Pharmacopoeia. In order to attain superior stability, the standard samples for calibration were kept at 4–8 ◦C for 5 days at most. Formic acid, metallic alcohol, and water originating from an Elga PureLab system, were used for the establishment of analytical conditions.

The determinations were performed on a Tripluquadrupol Access Max mass spec- trometry system and chromatographic separation on Transcend TLX 1 trip system. The sample centrifugation was done on a Hattick 4 centrifuge. Chromatographic separation was performed on a Phenomenex Kinetex C18 chromatographic column of 50 mm in length, internal diameter of 4.6 mm and particle size of 5 μm. The development of the analytical method was done in two stages: establishing working conditions on the mass spectrometry system and chromatographic optimization.

• Statistical Analysis Data gathered were registered, stored and statistically analyzed. The statistic process- ing of collected data was done in SPSS 24.0 using the Kolmogorov-Smirnov test for samples, the Pearson correlation coefficient, the ANOVA test, Wilcoxon rank comparison test and linear regression. Results with a value of $p < 0.05$ were considered statistically significant.

1.4.3.3 *Results*

The highest frequency regarding the oncology diagnostic was colon cancer (34.2% of total cases) followed by pulmonary cancer (24.1%), pancreatic and rectal cancer (13.8%) and esophageal cancer and liposarcoma (6.9%). All patients were diagnosed with stage IV cancer which means advanced or metastatic cancer.

Oxaliplatin treated cases represented the highest frequency (62.1%), followed by gemcitabine (20.7%) and cisplatin (17.2%) as seen in Table I.1.12.

A number of 22 out of 29 (75.9%) patients presented adverse effects after chemother- apy of which the most frequent were nausea (34.5%), loss of appetite, alopecia (27.6%), mucositis, xerostomia, vertigo, prickling sensations and fever (13.8%).

The highest values of GI score of 3 were present more frequently on tooth 16 (15.4%), followed by 21 (7.4%) and 41 (3.7%). Oppositely, the lowest values were registered on 36 where a score of 1 represented 20%, as observed in Figure I.1.12.

Similar to the GI results, the GBI showed the highest values at 16 (score 5–15.4%), followed by 21 (score 4–7.4%) and 41 (score 4–3.7%) and the lowest values were present at 36 with a score of 0—20%, as presented in Figure I.1.13.

Table I.1.12. Lot distribution regarding chemotherapy treatment, dosage, frequency and cancer types

Chemotherapy Agent	Number of Cases (%)	Mean Dose (mg)	Frequency of Administrations (Days)	Cancer Types
Cisplatin	5 (17.2)	48	21	Pulmonary
Oxaliplatin	18 (62.1)	181.44	14 (N = 5 cases) 21 (N = 8 cases)	Esophagus, Colon, Rectum, Pancreas
Gemcitabine	6 (20.7)	1699.33	7	Pulmonary, Pancreas, Liposarcoma

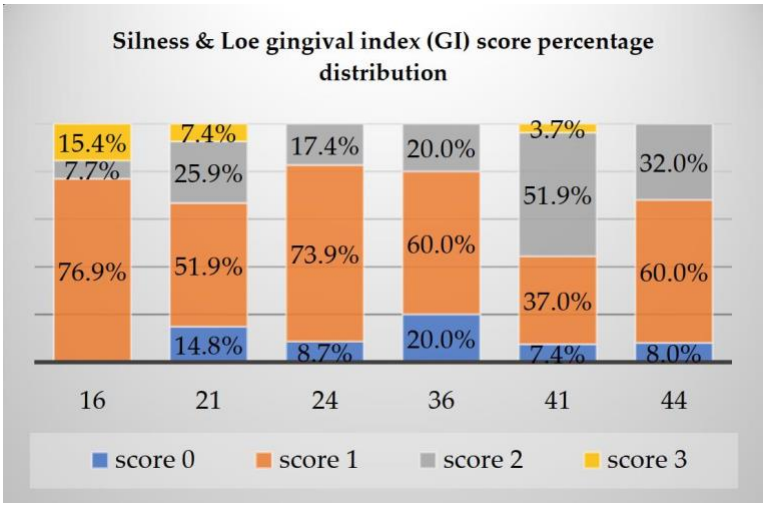


Figure I.1.12. Sillness and Loe gingival index mean score percentages distributions on Ramfjord teeth (teeth numbers 16, 21, 24, 36, 41 and 44).

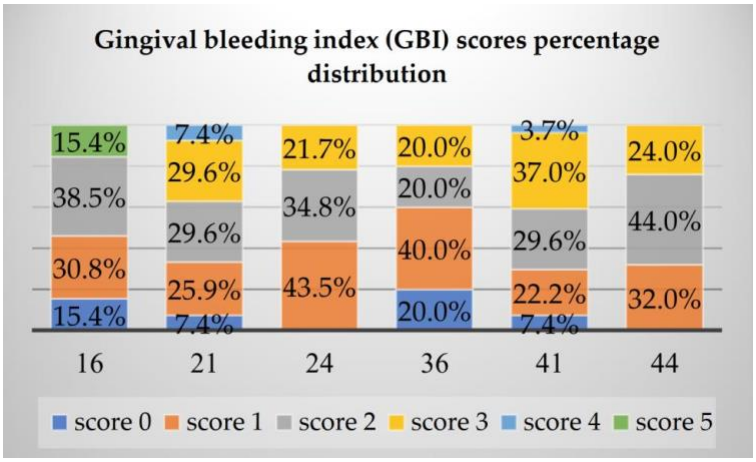


Figure I.1.13. Gingival bleeding index mean score percentage distributions on Ramfjord teeth (teeth numbers 16, 21, 24, 36, 41 and 44).

The CPITN index presented the highest values at 41 (score 3–14.8% and score 2–48.1%) and 44 (score 2–64%) with treatment needs of plaque and calculus removal, oral hygiene instructions and scaling and root planning.

The highest values of PD were found at 16 with a mean value of 1.611 mm and 36 with a mean value of 1.464 mm, thus the highest values were found in the lateral areas. Table I.1.13. shows higher overall mean values in the maxilla rather than the mandible.

Table I.1.13. PD values on Ramfjord teeth.

Probing Depth (PD) on Tooth Number (Ramfjord)	**N**	**Mean**	**Mean Standard Error**	**Standard Deviation**	**Minimum**	**Maximum**
16	13	1.6115	0.20165	0.72707	1	3.16
21	27	1.3844	0.11746	0.61035	0.58	2.91
24	23	1.3574	0.09628	0.46174	0.83	2.5
36	10	1.464	0.11756	0.37176	1.08	2
41	27	1.2863	0.14588	0.75802	0.58	2.83
44	25	1.2896	0.13483	0.67416	0.83	3.08

CAL mean values, on the other hand, showed highest values on the mandible where 36, 41 and 44 presented mean CAL values of over 3 mm, as seen in Figure I.1.14.

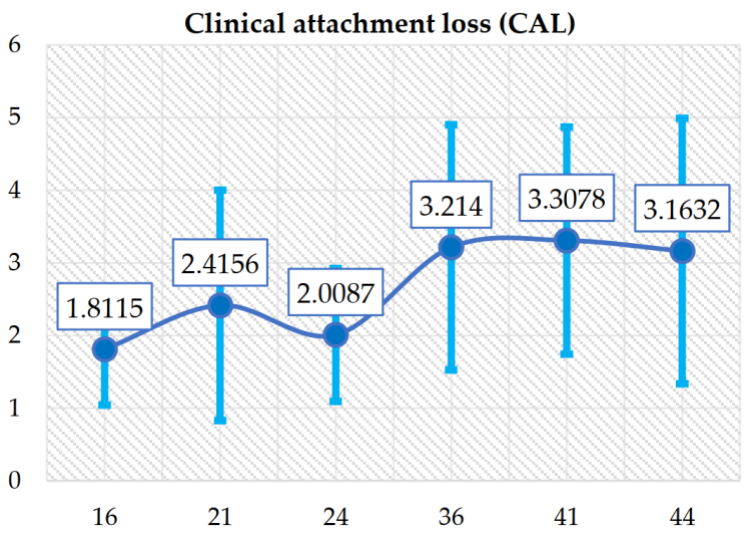


Figure I.1.14. CAL mean value distribution on Ramfjord teeth.

Determining the concentration of chemotherapy in saliva showed a similar curve for all three agents included in this study, following the same pattern. Lowest concentrations were found in T0, cisplatin and gemcitabine being absent in the saliva samples (OXA T0 = 0.0272 µg/mL) and highest concentration values were found in T1 (CIS T1 = 0.0016 µg/mL, OXA T1 = 0.0507 µg/mL and GEM T1 = 0.0106 µg/mL). After two hours after administration, all three chemotherapy drugs showed a decrease in saliva concentration, as observed in Figure I.1.15.

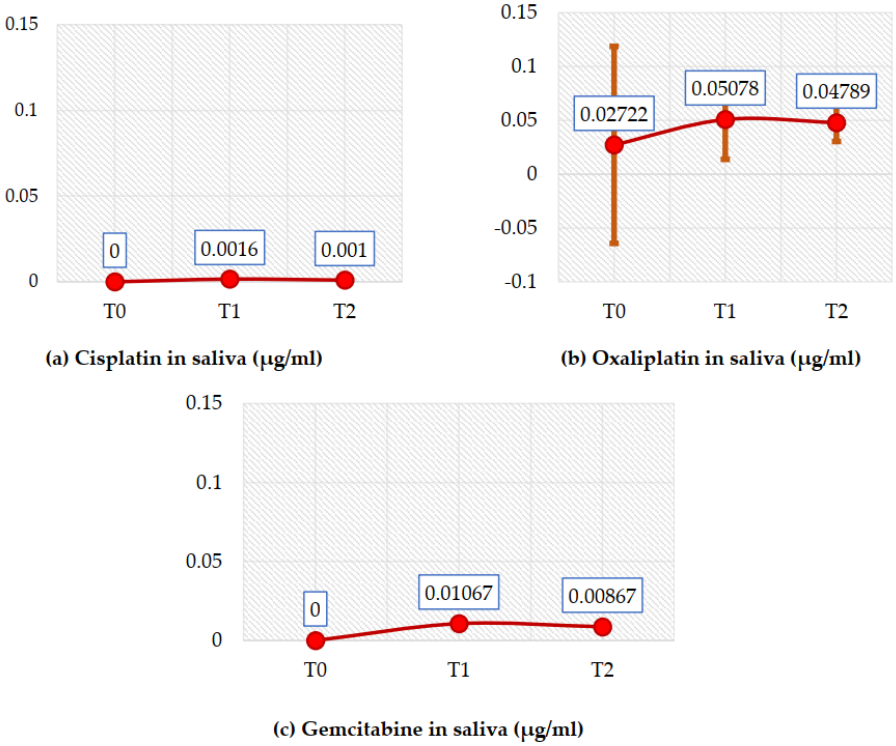


Figure I.1.15. Mean values of chemotherapy drugs detected at T0, T1 and T2 in saliva. (a) Mean values of cisplatin concentration (µg/mL); (b) mean values of oxaliplatin concentration (µg/ml); (c) mean values of gemcitabine concentration (µg/mL).

Using the Wilcoxon test for ranked comparison we compared the overall chemotherapy concentrations in T0, T1, and T2 and obtained statistical significance, as shown in Table I.1.14.

Table I.1.14. Wilcoxon test for chemotherapy concentration comparison in T0, T1, and T2.

		N	Mean Rank	Rank Sum	Z Statistics	p Significance
Saliva chemotherapy in T1 compared with T0	Negative ranks	2	28.50	57.00	−3.475	**0.001, SS**
	Positive ranks	27	14.00	378.00		
	Pairs	0				
	Total	29				
Saliva chemotherapy in T2 compared with T0	Negative ranks	2	26.50	53.00	−3.277	**0.001, SS**
	Positive ranks	25	13.00	325.00		
	Pairs	2				
	Total	29				
Saliva chemotherapy in T2 compared with T1	Negative ranks	27	14.00	378.00	−4.546	**0.000, SS**
	Positive ranks	0	0.00	0.00		
	Pairs	2				
	Total	29				

We then analyzed the correlation between the GI, GBI, CPITN, PD, and CAL and quantity of chemotherapy found in saliva in T0, T1 and T2 and found no statistical significance.

1.4.3.4 *Discussion*

Plasmatic determinations can be done for both cisplatin forms (protein bound or free circulating) (Sandler et al., 1999), but also for the total plasmatic platinum quantity. Plasma levels of cisplatin become undetectable after 24–25 h post administration where the infusion duration was 0.5 h, as was the case in the present study, and highest concentrations in plasma were obtained in the first hour post administration the peak being at 30 min for both bound and free cisplatin (Shelley et al., 2012).

The lowest concentration values determined in saliva in this present study were registered in T0, the highest were found to be in T1 right after administration at 30 min and then lowered again at 2 h (T2) after the administration was completed. These results confirm the presence of chemotherapy in the oral cavity and saliva in small quantities that follow a value curve similar to the plasmatic curve, and in the case of oxaliplatin it can still be detected in saliva even after a longer period of time, such as the end of the cycle between two administrations in our current study and coincides with our determinations in T0.

Clinical evaluation, which included the GI, GBI and CPITN indexes, alongside PD and CAL, partly reflected the level of hygiene and also offered a detailed view of the periodontal status in chemotherapy patients. Even though we did not achieve statistically significant results in correlating the presence of chemotherapy concentrations found in saliva with periodontal status, it has been stated in the literature that during chemotherapy there is a higher chance of infections occurring locally that can extend systemically (El–Housseiny et al., 2007; Chaveli-Lopez 2014). Moreover, it has been proven that chemotherapy can influence the salivary immunoglobulins by decreasing IgG and IgA levels which in turn can partly explain the patient's susceptibility to oral

infections (Epstein et al., 2002). The oral microbial community is influenced by chemotherapy as well as some saprophytic bacteria can become aggressive due to a decrease in granulocytes and increased fragility of the oral mucosa (Solomon et al., 2018).

As necrotizing gingivitis is one of the more frequent forms of periodontal manifestations, it has been shown that certain bacteria, such as *Prevotella*, *Fusobacterium, Actinobacillus, Actinomycetemcomitans* and *Actinomyces*, are associated with infections occurring in patients receiving chemotherapy (Mosel et al., 2011). On the other hand, it was shown in the past that periodontal status can influence the microbial composition during chemotherapy (Reynolds et al., 1989).

The most important periodontal modification was observed on the mandible in our study, where the most extensive losses were registered at 36, 41, and 44 (mean CAL = 3.214, 3.308, and 3.163 mm, respectively). Radiotherapy has proven to have a similar effect, which registered 92% clinical attachment loss on the mandible; the loss being even greater in cases in which radiotherapy was aimed at this region (Marques 2004). Analyzing the CAL level offered additional data regarding the periodontal status of chemotherapy patients (overall mean value of CAL = 2.902 mm). From our observations and statistical analyses, we have determined that the progression pattern of periodontal disease in chemotherapy patients manifests more frequently with recessions rather than periodontal pockets.

The overall mean value of the Sillness and Loe GI was 1.265 in our study, which suggests the presence of minimal gingival modifications with the exception of a low number of patients, in whom a moderate or severe level of inflammation was observed. We also observed that the highest scores for the GI and GBI were found in the maxilla, especially in the lateral areas. During chemotherapy, the periodontal inflammation level is increased and, at the same time, so did the GI, which offers a general view on clinical periodontal status (Djuric et al., 2010). Under normal circumstances, the level of gingival affliction is dependent on bacterial plaque quantities and level of oral hygiene. It has been indicated in the literature that, during chemotherapy, periodontal inflammation can be exacerbated, even if the oral hygiene levels are good (Ohrn et al., 2001).

1.4.3.5 *Final remarks*

Our study demonstrates that a significant fraction of systemically administered chemotherapy can be found in the oral cavity and saliva of oncologic patients. Oxaliplatin is identified more easily in oral fluids compared to other studied chemotherapeutic agents. The maximum concentrations for cisplatin, oxaliplatin, and gemcitabine were significantly quantified 30 min after the completion of administration.

Further research is required in order to determine the effect of chemotherapy on periodontal tissues and its impact on the prognosis of periodontal disease.

1.4.4. Periodontitis and coronavirus disease

1.4.4.1 *Introduction*

According to data provided by the World Health Organization (WHO), the new coronavirus, SARS-CoV-2, had caused 5,493,846 deaths globally by 10 January 2022, of which approximately 20% were recorded in the USA. Multiple factors are responsible for differences in contamination and mortality due to SARS-CoV-2 between countries. These include the implementation of domestic policies to control the spread of infection, vaccination, population density, comorbidities and the proportion of the ageing population, to name a few (Devlin and Soltani, 2021). This has led to a significant variation in the degree of infectiveness and mortality (Tables I.1.15 and I.1.16) (Darvish and Salman 2021).

Table I.1.15. SARS-CoV-2 infection rate by August 2021 (Darvish and Salman 2021).

SARS-CoV-2	Percentage
United States	18.19%
India	15.22%
Brazil	9.65%
Russia	3.17%
France	3.11%
United Kingdom	3.06%
Spain	2.25%
Romania	6.87%
Average	7.69%

Table I.1.16. SARS-CoV-2 cases and mortality rate by 10 January 2022

Country	Cases Confirmed	Deaths	Case Fatality (%)
Peru	2,358,685	203,019	8.6%
Brazil	22,529,183	620,251	2.8%
Belgium	2,231,686	28,459	1.3%
Italy	7,436,939	139,038	1.9%
Mexico	4,125,388	300,334	7.3%
United States	60,074,429	837,594	1.4%
United Kingdom	14,563,769	150,634	1.0%
Ecuador	559,950	33,699	6.0%
Romania	1,844,537	59,011	3.2%
Spain	7,164,906	89,934	1.3%
Portugal	1,499,976	19,029	1.3%
France	12,218,022	126,427	1.0%
South Africa	3,526,054	92,453	2.6%
Iran	6,206,405	131,878	2.1%
Russia	10,470,006	309,787	3.0%
Greece	1,507,616	21,394	1,4%
Austria	1,339,421	13,848	1.0%
Germany	7,553,743	114,033	1.5%
Average	970,464,876	163,519,323	2.70%

Among medical practitioners, dentists and dental staff have an increased risk of being infected with airborne pathogens such as SARS-CoV-2 because they are always exposed to droplets and aerosols produced during specific treatment procedures. Transmission may occur due to the inhalation of droplets and aerosols from an infected individual or by direct contact with mucous membranes, oral fluids and contaminated instruments or surfaces. To evaluate the effects of intraoral and extraoral aspiration on the spread of infection during dental treatments, the bacterial colonization of droplets and aerosols was evaluated following simulations of scaling by the dentist and dental hygienist in three healthy volunteers. Extraoral aspiration has been shown to reduce the production of droplets and aerosols, and since it is restricted to the left and back of the dental chair, right-handed operators could perform treatment with relatively low contact with the pathogens. This study suggests that both aspiration methods were effective; however, extraoral aspiration was more effective in reducing the number of droplets and aerosols compared to intraoral aspiration or a lack of aspiration (Senpuku et al., 2021). Furthermore, it has been shown that saliva represents a potential source of contamination for many patients. This aspect is of critical importance in public health management, not only for SARS-CoV-2, but also for other pathogens, considering the high rate of exposure to saliva by dental professionals (Xu et al., 2020).

Since COVID-19 is primarily spread through droplets and aerosols, it could reasonably be assumed that dentistry might be among the professions with the highest mortality rate (Devlin and Soltani 2021. However, when the number of deaths was examined in England and Wales between March and December 2020, there was no evidence of a higher mortality rate among dentists caused by COVID-19. This led to the conclusion that the low infection rate of dentists might be due to the rigorous safety protocols implemented. The American Dental Association (ADA), as well as most European dental organizations, recommends patient prescreening before visiting the clinic, allowing only one patient at a time in the waiting room, measuring staff and patients' temperatures, hand washing and sanitizing, access to sanitizers for patients, disinfection of surfaces, personal protection equipment for the medical team, disposable shoe covers for patients, use of UV lamps and other air purifiers and high-efficiency aspiration during treatments (Figure I.1.16) (Devlin and Soltani 2021; Giraudeau 2021). For example, Butera et al. suggested the use of the bio-inspired systems in nonsurgical periodontal treatment in order to reduce the risk of bacteremia and aerosol generation and improve clinical, microbiological and immunological parameters by decreasing bacterial load (Butera et al., 2020).

This qualitative, narrative review summarizes the most recent literature on the effects of the COVID-19 pandemic on dental practice and dental education, as well as the use of teledentistry in the delivery of oral and dental care to avoid virus contamination. Research and review papers were identified and selected using Scopus, PubMed and Web of Science scientific databases. Commentaries, letters and in vitro studies were excluded from the analysis. The paper describes in a comprehensive and critical manner, the effects of COVID-19 pandemic on the delivery of oral and dental care and dental education and its impact on current and future dental practice.

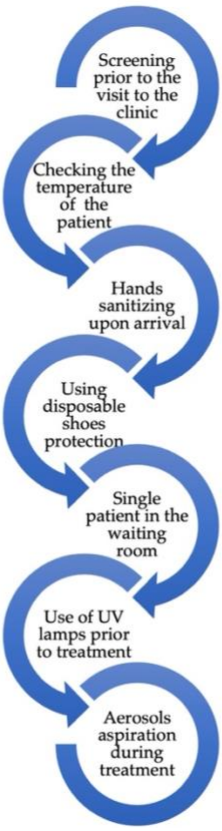


Figure I.1.16. Safety protocol for dental patients during the COVID-19 pandemic.

1.4.4.2 *COVID-19 and the new approach to dental healthcare*

Teledentistry has been defined as "the remote practice of dentistry by oral health professionals, within the limits of their practices, via the use of information and communication technology" (Butera et al., 2020). Its objectives should not depart from those of in-person care, and may include diagnosis, prevention and post-treatment monitoring, specialist advice, treatment, prescription, referrals and other practices. Approximately 80% of dentists have adopted precautionary recommendations and modified them according to the type and particularities of each dental treatment (Kwok et al., 2020). For example, to increase the safety of the working team, a recent study showed that approximately 30% of dentists wore additional protective equipment, applied sanitation and ventilation procedures beyond those recommended by the guidelines and local health authorities, preferred to treat infected patients or those suspected of infection at the end of the working day and used an FFP3 mask during treatment. Approximately 78% of dentists replaced the FFP2 mask after eight hours of use, even when treating non-contaminated patients, and 62% covered the FFP2 mask with an FFP1 surgical mask (Kwok et al., 2020).

Furthermore, 89% of dentists recommended oral rinses with solutions based on hydrogen peroxide and chlorhexidine before commencing therapeutic procedures. The combination of hydrogen peroxide with chlorhexidine solutions has been shown in vitro to be more effective than either solution alone in preventing transmission of SARS-CoV-2 (Kwok et al., 2020). Other

studies have shown a decrease in salivary viral load after a 30 s mouth and oropharynx gargle with 15 mL of 1.5% or 3% hydrogen peroxide solution or 0.12% chlorhexidine solution (Salgarello et al., 2021). Likewise, brief (30 s) rinses with 0.2%, 0.4% or 0.5% povidone–iodine (9 mL) or 0.05% cetylpyridinium chloride (15 mL) have been proven to be effective. Similar effects were obtained with cetrimide rinses in oncologic patients. However, the degree to which these solutions are effective in preventing or decreasing SARS-CoV-2 contamination risks, particularly in vulnerable populations, still need to be examined (Vergara-Buenaventura and Castro-Ruiz 2020).

Although SARS-CoV-2 has a predominantly airborne transmission, salivary contamination can be controlled much easier in dental offices. For example, recent studies that examined increasing suction capacity by using a large volume of air (150 mm Hg or 325 L/min) suggest that this measure may be sufficient to eliminate viral contamination. To further increase safety at work, Italian dentists have adopted, as a preventative measure, ventilation of dental treatment rooms after examination of each patient, regardless of the dental treatment performed. In rooms with poor mechanical ventilation, portable air filters with a high-efficiency particulate air filter (HEPA) have effectively reduced aerosol accumulation and accelerated aerosol removal. Taken together, these studies show that new measures put in place in dental offices significantly mitigate the risks of SARS-CoV-2 contamination, and the risk of contracting COVID-19 in the dental office is relatively low (Scarano et al., 2020).

Dental Public Health Issues during the COVID-19 Pandemic

A recent study conducted in Italy showed a state of normalcy in dental practices after the initial wave of COVID-19 pandemic. Since its onset, Italian dentists have experienced high levels of anxiety and stress, mainly due to the rapid spread of SARS-CoV-2 at the national level and the need for rapid adaptation to the new health standards in dental offices. Approximately 80% of Italian dentists resumed their regular activity after the first quarantine. However, there were some geographical differences due to the evolution of the virus over time. For example, the reopening rate of dental offices ranged from 36% in the United Kingdom to 47% in Palestine, while in Italy and the USA this figure reached 99%. Approximately 80% of dentists have adopted preventative measures and adapted them to specific dental treatments. Notwithstanding these changes, dental offices incurred significant financial losses of over 70% due to the COVID-19 pandemic. In a survey conducted by the British Dental Association, 70% of dental clinics reported that they could only remain viable and maintain their usual number of employees for up to three months (Kwok et al., 2020).

Pre-, during and Post-Pandemic Particular Aspects of Dental Treatments

A recent study highlighted the changes in the spectrum of procedures performed before and during the pandemic. For example, during the COVID-19 pandemic, the number of conservative procedures, such as coronal restorations or root canal fillings, decreased significantly, while the percentage of surgical procedures increased significantly. In the following months, the decrease in the number of patients was offset by an increased number of procedures per visit (Meethil et al., 2021; Nijakowski et al., 2021).

Likewise, several changes were recommended when performing various treatment procedures. For example, the mechanochemical treatment of carious lesions was carried out using hand tools instead of rotary ones. Similarly, for periodontal treatments, manual scaling was chosen over ultrasonic scaling. In cases of symptomatic irreversible pulpitis, biological methods, such as pulpotomy or pulpectomy, were recommended as much as possible (Benzian et al., 2021).

On the other hand, in patients with extensive destruction of the hard dental tissue accompanied by severe pain, it was necessary to opt for the extraction of the affected tooth. Thus, it was possible to reduce the risk of infection, shorten treatment time and minimize repeated visits. In the case of excessive bleeding, multiple extractions or other oral surgeries, resorbable sutures were preferred. Specific interventions have been performed in pediatric patients to reduce aerosol-generating procedures and use non-invasive or minimally invasive methods. For example, fissure sealing, the topical application of varnishes and resin infiltration using the ICON method, were chosen in order to stop the evolution of non-cavitary carious lesions. At the same time, to minimize virus transmission and contamination, there was an increase in the number of certain procedures, such as indirect capping, atraumatic restorative treatment, provisional therapeutic restorations, the Hall technique and the application of diamine silver fluoride (Nijakowski et al., 2021).

1.4.4.3 *Teledentistry and COVID-19*

Telemedicine has proven to be an effective tool in mitigating some of the effects caused by the imposition of restrictive measures during the COVID-19 pandemic. Several authors have suggested that the lack of coherence in the implementation of telemedicine as a solution for continuous medical education is one reason for the absence of uniform protocols for aerosol-generating procedures (Benzian et al., 2021). For example, dentists in the UK changed the way they approached clinical cases and developed a triage system using remote consultations. The treatment was limited to advice, analgesia and first-line antimicrobial therapy, with the goal of reducing the risk of transmission. COVID-19-positive patients, confirmed by RT-PCR, were directed to in-person treatment only in emergency dental centers, which have been previously authorized for this purpose (Devlin and Soltani 2021).

Teledentistry has been increasingly used by dental schools during the pandemic. Although there are regional differences in isolation policies, the severity of the outbreak and the availability of resources have greatly impacted the functioning of dental schools during the pandemic, although the responses of dental institutions to the COVID-19 pandemic show some similarities. For example, distance learning was the only alternative to theoretical dental education in many institutions. While e-learning already existed, it evolved and expanded as a result of the COVID-19 pandemic, with the use of synchronous online teaching methods when interacting with participants (Expósito-Delgado et al., 2021). In Italy, for example, telemedicine played a key role in reducing the spread of COVID-19 from the start of the pandemic. A number of teledentistry platforms such as OloHealth® have emerged since 2019 and are dedicated to the prevention and

management of oral malignant disorders in addition to improvement of oral health, in order to reduce unnecessary travel and loss of productivity (Ghai 2020).

Likewise, teledentistry has been used successfully when treating patients with more complex oral pathologies by carrying out photographic teleconsultations for the first visits and for subsequent evaluations, thus ensuring a good remote patient management (Figure I.1.17). After an adequate anamnesis by videocall and photographic evaluations during the first visit, patients were followed up with remote evaluations of their pathologies, such as fungal infections, dry mouth syndrome, sialolithiasis, traumatic ulcers, third molar pericoronitis and others (Giudice et al., 2020).

Telemedicine was further used in cases that would normally require clinical examination in order to distinguish between potentially malignant lesions from those that were truly malignant and necessitated immediate attention. This allowed dentists to keep patients with precancerous lesions, osteonecrosis of the jaw associated with medication and autoimmune diseases under control by comparing recently received photos with the last photos taken at the dental clinic. For these pathologies, clinical changes were evaluated to determine the risk of malignant processes and manage possible recurrences, infections, pain and stability of lesions (Ghai 2020).

For example, Machado et al. emphasized the importance of oral telediagnosis when examining a 49-year-old female patient with controlled diabetes and symptomatic pinkish nodular lesions affecting the buccal mucosa, associated with purple spots on the skin. The dentist took photos with a short description using the WhatsApp platform and recommended a hematological examination based on idiopathic purpura. Severe thrombocytopenia was confirmed, and the patient was referred to the hospital for specialized systemic treatment with steroid medication (Dusseja et al., 2020). When teledentistry is not available or cannot be used, saliva tests can be employed as a solution for screening patients with minimal physical contact given the strong link between salivary diagnosis and oncologic pathology (Machado et al., 2020).

Notwithstanding, teledentistry appears to be a promising tool in the remote management of patients requiring non-surgical or surgical treatment, especially by reducing costs and waiting times. To this end, patients that need regular treatment for chronic conditions, geriatric patients or those with special needs, and patients living in remote, less-accessible areas can benefit the most from teledentistry. This can lead to reduction in the number of visits to the dental office, shorter waiting times, decreased no-show appointments, and reduced unnecessary exposure of healthy patients. The efficacy of teledentistry has mostly been studied in pediatric dentistry, oral medicine, orthodontics and periodontics for several procedures and with various degrees of success. For teledentistry to be effective, it requires an educated patient not only familiar with the new digital technologies (i.e., taking high-quality intraoral digital images, data storage, virtual communications using various apps, etc.) but also with basic dental knowledge (Estai et al., 2016). For example, knowledge of the management of orthodontic appliances or teeth eruption, which represent a significant portion of emergency visits, can reduce office visits by approximately 20 percent. Fixing a loose or poking orthodontic wire or appliance, smaller fractures or tissue trauma can all be handled by teledentistry and avoid in-person visits.

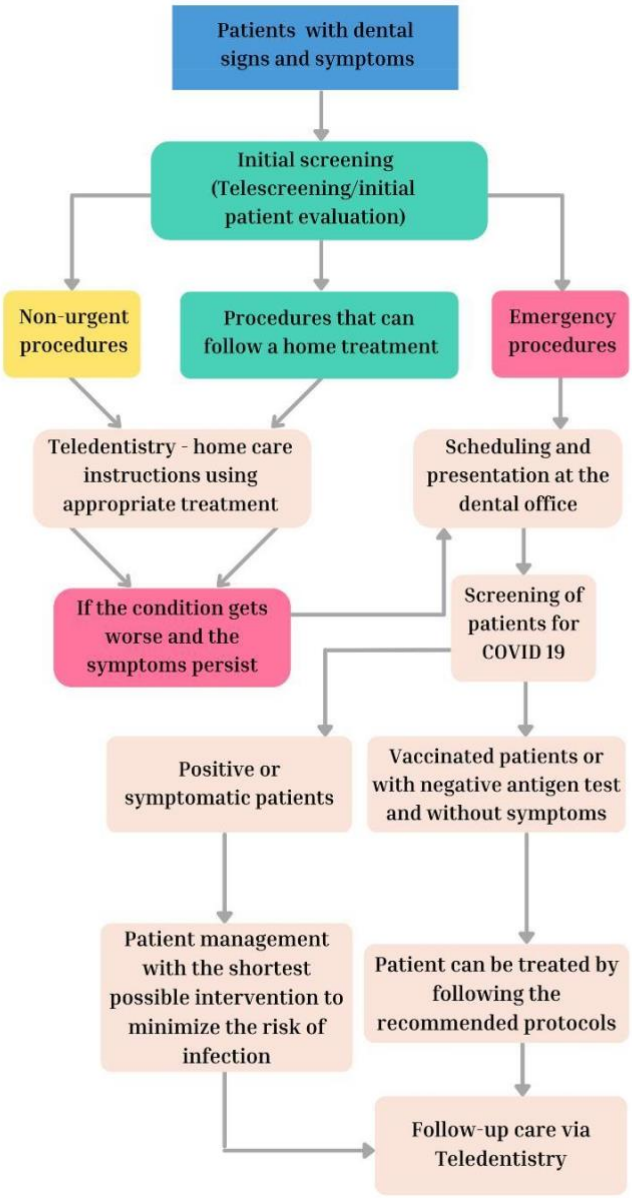


Figure I.1.17. Management of dental protocols during COVID-19 pandemic.

Indeed, some studies have shown that nearly half of all dental emergencies can be managed utilizing teledentistry. However, some of these procedures such as chronic pain, dental caries, severe trauma, fractures and orthodontic treatments would need to be evaluated frequently to avoid unnecessary delays that may lead to later complications such as infections. Not all dental practices and specialties are prepared or have the expertise for successful use of teledentistry, and it requires significant resources and infrastructure. For this reason, there is a wide range of teledentistry applications and its use varies across countries, regions, dental specialties and

offices. Irrespective of these differences, the most important advantage lies in triaging patients, reducing the number of site visits and, in doing so, reducing exposure to pathogens such as SARS-CoV-2 (Kappenberg-Nitescu et al., 2021).

In addition to the various devices that can be used in telemedicine, instant messaging applications have become increasingly popular for better communication between doctor and the patient. For example, WhatsApp-based teleradiology consultation proved to be an effective tool for interpreting X-rays with different dental pathologies (Madi et al., 2021). Furthermore, dental telemedicine can be successfully applied during the follow-up of patients who have undergone oral and maxillofacial surgery, although more work is required to determine patients' compliance and doctors' attitudes towards integrating remote dentistry in the standard protocols of telemedicine (Torul et al., 2021).

The Impact of COVID-19 on Dental Staff

It is well established that COVID-19 had a significant impact on nurses working in the field of dentistry, which affected the quality of medical services. Chronic diseases, immunodeficiencies, the risk of SARS-CoV-2 infection, working in a private environment and family responsibilities associated with financial risks caused by the pandemic have all contributed to significant increases in anxiety, burn-outs and other mental health disorders (Wallace et al., 2021). This necessitates collective actions at the government level and a set of measures that can contribute both to the prevention and treatment of these manifestations (Mekhemar et al., 20121).

Among dental staff, dental hygienists have been greatly impacted by the pandemic given the high risk of occupational contamination via aerosol loading due to their work in the maintenance of periodontal health and prevention of dental diseases. Although there are differences in the application of working protocols of prophylaxis, for assistants working in the private sector compared to those working in the public health system, the emphasis has been on minimizing the use of aerosol-generating procedures. The existence of protective equipment for dental teams, an adequate infrastructure as well as the correct management of patients, all contributed to an increase in trust and safety within medical teams. For example, a recent study conducted in the Czech Republic showed that well-chosen anti-epidemic measures adopted by dental professionals can reduce occupational infection risks associated with SARS-CoV-2 (Schmidt et al., 2021). On the other hand, other studies examining the knowledge, attitudes and practices of the Turkish pediatric dentists, showed a satisfactory level of knowledge regarding COVID-19 prevention, although infection control measures could have been better implemented (Koc et al., 2021).

1.4.4.4 *The impact of COVID-19 on dental academic environments*

Emotional and Psychological Effects

Academics working in the field of dentistry have been subjected to a high level of stress during the COVID-19 pandemic, not only related to teaching and research activities but also to concerns related to the possibility of contaminating their family members (Morgado et al., 2021). This triggered an immediate and acute need for developing and implementing psychological

support measures to reduce the level of mental stress among members of the academic staff. For example, Balkaran et al. recommended the use of meditation, specialized counselling and holding seminars for health promotion as therapeutic measures. Similarly, a plethora of measures have been proposed for dental students, given the critical role of mental health in the educational evolution and behavioral development of dental students preparing for medical careers .

Chronic cardiovascular diseases, smoking and being female, as well as the economic impact of SARS-CoV-2 on the dental profession, have been shown to negatively affect the psychological status of dental students (Balkaran et al., 2021; Mekhemar et al., 2021).

The COVID-19 pandemic has led to an increase in negative emotional states among students. Students were the population group most affected by the pandemic, showing an increased prevalence of stress, anxiety and insecurity (Zarzecka et al., 2021). Female students were found to be affected more than males, with a high risk for developing depression and negative emotional states, which was associated with an increase in leisure time and decreased physical activity (Mekhemar et al., 2021). Therefore, examining these factors can play a key role in developing public health policies to minimize the psychological impact among future dentists.

Quality of Dental Education

The COVID-19 pandemic has considerably affected the quality of dental students' training. Dentistry is a scientific–educational field which combines theoretical concepts and principles with the acquisition of practical skills. In the absence of a mandatory residency program, basic dental education requires sufficient preclinical and clinical training to ensure an adequate level of competence for future professionals in the field.

While distance learning could be a commonly adopted strategy for higher education in various fields, a unique challenge for dental education is the dependence on the requirements of clinical experience to achieve minimal competency in performing dental treatments. Since many dental procedures produce considerable amounts of aerosols and droplets, many routine and elective dental treatments were suspended during the pandemic, thus affecting the training of dental students (Hassan and Amer, 2021; Talapko et al.,2021).

Dental Research

The COVID-19 pandemic has greatly impacted research in the dental field. In some cases, having limited access to patients delayed and even compromised the results of clinical trials and restricted the implementation of new ones. For example, saliva and crevicular fluids are valuable diagnostic tools in dental medicine. Diagnostics based on salivary matrix metalloproteinases (MMPs) that can successfully quantify periodontal inflammation should be cautiously used because of potential contamination risks (Wang et al., 2002; Sabino-Silva et al., 2020).

To overcome these hazards, other methods have been employed, such as the finite element method (FEM), which uses mathematical models to simulate clinical reality and does not involve patient contact. FEM proved to be an extremely useful and reliable alternative option during the COVID-19 pandemic by providing optimal prognoses and validating different protocols of

treatment in various dental specialties, such as periodontics, orthodontics or prosthodontics (Devlin 2021).

The oral cavity seems to play an important role in coronavirus disease 2019 pathogenicity. (Huang et al., 2020; Iranmanesh et al. 2020; Dziedzic and Wojtyczka 2021). They have been found on the epithelial cells of the tongue, oral mucosa, salivary glands, gingiva, and periodontal pockets at levels comparable to those in the lungs and tonsils.

In addition, severe acute respiratory syndrome coronavirus 2 can be identified in oral fluids and saliva, and its oral viral load has been associated with disease severity (Iranmanesh et al. 2020).

Some unspecific oral lesions have been associated with coronavirus disease. These include dry mouth, oral vesiculobullous or pustulous lesions, lip necrosis, fissured or depapillated tongue, or erythematous or hemorrhagic mucosal lesions (Iranmanesh et al. 2020). Such lesions are mostly found among patients with systemic conditions that involve some degree of immunosuppression. (Dziedzic and Wojtyczka 2021).

Relevant risk factors associated with coronavirus disease severity, including smoking, increased age, obesity, diabetes, hypertension, and cardiovascular disease, are also significantly associated with periodontitis.

Hence, it is uncertain whether these factors could just behave as comorbidities or whether there are specific mechanisms and pathological pathways linking periodontitis and increased coronavirus disease severity (Shenkein et al., 2020). The association between coronavirus disease 2019 infection and periodontitis has also been investigated in two retrospective studies (Sirin and Ozcelik 2021). Other studies have also suggested a possible effect of coronavirus disease infection on periodontal health (Katz et al., 2020).

It has been predicted that an increased prevalence of acute periodontal lesions, particularly necrotizing periodontal disease, could arise in association with coronavirus disease–confirmed cases (Patel and Woollley 2021). However, this hypothesis is yet to be confirmed.

It is well established that translocation of periodontal pathogens to blood (eg, bacteremia) and the associated systemic inflammation are mechanisms contributing to the links between periodontitis and systemic diseases, such as diabetes, cardiovascular diseases, and rheumatoid arthritis (Genco and Sanz 2000). However, these mechanisms have not been clearly demonstrated in the association between periodontitis and increased coronavirus disease severity.

Severe acute respiratory syndrome coronavirus 2 in the periodontal pockets A study on cadaver biopsies from coronavirus disease–positive patients has reported the presence of the severe acute respiratory syndrome coronavirus 2 within their periodontal tissues (Fernandez Matuck et al., 2020). This has led to the hypothesis that periodontal pockets may serve as reservoirs for severe acute respiratory syndrome coronavirus.

It is well established that periodontal pockets present an ideal environment for harboring biofilms rich in bacterial and viral species that may invade the tissues through the frequently ulcerated pocket epithelium (Badran et al., 2020; Raisanen et al., 2020).

This pathogenic environment could facilitate the entrance of the severe acute respiratory syndrome coronavirus 2, either directly through this damaged epithelia or indirectly by the

upregulation in the expression of angiotensin-converting enzyme 2 receptors induced by some periodontal pathogens, such as Fusobacterium nucleatum (Rpganovic et al., 2021). Systemic inflammation in periodontitis is characterized by high levels of C-reactive protein and proinflammatory cytokines (interleukin-1 and IL-6) that have been associated with initiating or aggravating diseases, such as diabetes and cardiovascular diseases.

Periodontitis has also been implicated with the release of neutrophil extracellular traps, an alternative form of cell death secondary to increased levels of mediators, such as interferon-alpha. (White et al., 2016; Fine et al., 2021). The adverse outcomes of coronavirus disease infections have also been associated with an uncontrolled hyperinflammatory reaction known as the "cytokine storm."

This condition involves increased serum levels of interleukin-2, 6, 7, 8, and 10, tumor necrosis factor-alpha, granulocyte colony- stimulating factor, interferon-gamma inducible protein 10 (IP-10), monocyte chemoattractant protein 1, macrophage inflammatory protein 1-alpha, galectin- 3, and C-reactive protein, with concomitant significantly lower numbers of T-lymphocytes (Mehta et al., 2020). This inflammasome, which has been shown to increase the serum and salivary of periodontitis patients, may play a significant role in the coronavirus disease cytokine storm and has been shown to aggregate in the lungs, resulting in fatal pneumonia (Toldo et al., 2021).

1.4.4.5 *Final remarks*

The COVID-19 pandemic has affected and still continues to significantly impact the delivery of dental healthcare due to changing clinical protocols and adapting them in order to minimize contamination risks. Teledentistry has expanded its initial scope and, when correctly implemented, could be an effective tool, but should be considered as a complementary means rather than an alternative to on-site, conventional treatments that are based on the principle of personalized medicine.

Teledentistry can offer tremendous benefits in the delivery of some applications, while it can be limited in others. For example, pre- and post-operative counselling, education and care, nutrition advice and quick access to images of oral cavities through user-friendly imaging devices accessible to patients can all be performed via teledentistry. On the other hand, there are many challenges associated with teledentistry. These are primarily related to the lack of guidelines, standardization and scientific validation of teledentistry procedures and tools used in addition to issues related to data security and privacy.

Other constraints are related to the inability to perform a clinical tactile exam, lack of direct contact with the patients, risk of misdiagnosis, lack of technological infrastructure, poor access to the Internet, lack of hardware, low information technology literacy, resistance to new technologies, and a lack of training and consumer awareness. There is no "one-fit-all" solution to overcome these challenges, and as the field evolves, new creative models will be developed in order to fit particular scenarios. Irrespective of the model used, the patient should be provided with the same quality of dental care as performed in the clinic. As a minimum, it should meet the following criteria: provide easy access to all populations, including the underserved, provide oral

health delivery, including specialty care in a timely manner, and be sustainable, affordable and time effective.

There are several limitations of this narrative review that include the inherent lack of quantitative analyses of published studies and being prone to biases. Notwithstanding, teledentistry proved to be useful in enhancing communication with, and treatment of, various categories of patients, such as those from nursing homes and prisons, and it is cost and time efficient. It is readily accessible, user friendly, and will no doubt continue to be expanded to new areas of dentistry and remote dental services, even after the COVID-19 pandemic.

Moving forward, teledentistry will play a significant role in dental education and curriculum delivery by using technological advances in offering the skills required to maintain the quality of care while minimizing disease transmission. Finally, there is a need for more long-term comprehensive studies evaluating the impact of teledentistry in prevention, clinical outcomes and delivery of treatment across all branches of dentistry, as well as in developing distant training protocols in order to provide dental education in a safe environment.

Dental staff, academic personnel, dental students and dental researchers were severely affected by the COVID-19 pandemic, which led to a decrease in the quality of care, clinical work and practical training. Despite its limitations, teledentistry has become a critically important tool during the COVID-19 pandemic in mitigating the risks of virus contamination and transmission. Overcoming challenges in adopting teledentistry by improving patient and management tools via new technologies coupled with innovations in dental engineering and equipment to minimize aerosol-transmitted pathogens will, no doubt, find the dentistry world better prepared to withstand the negative impact of a potential future pandemic.

Thus, future research should focus on improving the quality and reliability of teledentistry in order to eliminate current technological errors and further integrating it as a complementary option in dental healthcare systems worldwide.

CHAPTER 2:
CURRENT TRENDS AND NEW HORIZONS IN PERIODONTAL DISEASE

1.5. State of the art

Magical, religious and herbal treatments were demonstrated in almost all of the early writings. However, methodical, carefully reasoned therapeutic approaches did not exist until the middle-ages and modern treatment with a scientific base and sophisticated instrumentation did not develop until the 18th century. Prior to the 1950s, diseases were mostly treated by root debridement and the extraction of the affected teeth. Until the 1970s, it was primarily the symptoms of periodontal diseases that were treated. The goal was radical elimination of the periodontal pocket (resective therapy). The means were gingivectomy, flap procedures and osseous surgery.

The disadvantages were the massive sacrifice of periodontal tissues, lack of regeneration and clinically elongated teeth. These disadvantages, along with the realization of the importance of aetiologic agents, raised questions about the necessity of total pocket elimination, and the control of subgingival infection by a thorough scaling and root planning (nonsurgical therapy), with and without antibiotics, became a commonly used treatment during the 1980s (Yilmaz et al., 1994).

Humans began to manifest periodontal symptoms due to aggravating oral hygienic conditions since beginning to eat processed food approximately 10,000 years ago. It is believed that tooth brushing habits in some regions began at the same time. Periodontal disease was first described 1,000 years ago in a book, in which the concept of a scaler was introduced along with techniques for removing dental calculus. Currently, scaling remains one of the major preventive and therapeutic for periodontal disease. Recently, however surgical treatment has also been employed and preventive vaccines have received intense investigation.

If you’re looking for the beginning of oral health care, you’ll have to start early. Even in Egypt, there were records of dental practices aimed at easing pain. Some say that evidence can be found of the earliest implants as well. Their methods sometimes seem like the cure was worse than the disease. Others, like dental extraction, are a relatively normal thing even today.

Chinese Medicine – China would use the earliest known toothbrushes in the world. Records indicate that dental extractions, and even implants, were used here for thousands of years. Conversely, it appears there must have been significant differences between social classes diets in the earlier phase of the Chinese history (Sakashita et al., 1997).

In Native Americans, many cultures demonstrated multiple approaches. One common theme in these cultures was the use of sage and other herbs as toothbrushes. There was even a form of rudimentary toothpaste made from the cucacua plant (Goldberg et al., 1976).

Colonial Era – In the colonial area in America, toothbrushes were unheard of. Instead, they’d chew sticks or use bones and feathers as toothbrushes. Some would opt to use salt as an abrasive to clean the surfaces of their teeth.

Island Nations – Archaeology of certain island cultures revealed skulls with seashells hammered into the jaw. Remodeling suggests that they worked, and the bone had had time to heal (Armitage 2000).

Modern dentistry began making real strides once we understood the role of bacteria in oral health. *Streptococcus mutans* were discovered to be the primary culprit in tooth decay. It later was determined to have a central role in gingivitis and periodontitis. With this discovery, we could aim our sites at eliminating the actual source of our problems.

Specifically, according to experts, the history of periodontics begins with the *Sumerians* and from there to the present day. And it is that already in the year 3,000 a.c it is known that in Mesopotamia oral hygiene was practiced. Proof of this are the decorated gold toothpicks that have been found in numerous sites in the area (Glickman, 1963).

An interest in the cleaning of the mouth that does not surprise us if we consider that periodontal disease was one of the most frequent in *Ancient Egypt*.

Hippocrates of Kos was a physician of *Ancient Greece* and who, through the ailments of his patients, devoted himself to identifying the causes of periodontal disease. He discovered that the inflammation of the gum could be due to accumulations of stones or, that gingival hemorrhage incurred in case of splenic diseases.

The history of periodontics also takes us to the years of the *Roman Empire* in which there were also important advances thanks above all to two names: Aulus Cornelius Celsus and Paul of Aegina. The latter, in fact, is credited with the creation of a medical encyclopedia consisting of 7 volumes in which oral surgery is already discussed (Savage-Smith 2011).

The history of periodontics you can not miss a stop in the *Renaissance* and is that the renewal in the sciences that took place at this time also had its effects on medicine in general and dental health in particular. It is in this century when a first identification and differentiation of the types of periodontal diseases is made or innovative techniques for the time such as gingivectomy begin to be used.

According to experts, modern dentistry was born in Europe and this is thanks to the fact that in this continent was “The father of dentistry” - Pierre Fauchard is known. It is known that this French doctor published books in which he described the anatomy and basic oral functions, collected the symptoms of most oral problems and put on the table different treatments to cure periodontal diseases.

To Fauchard, we also owe many of the treatments that we continue to use in dentistry today from the invention of the filling as a treatment for cavities to the first dental appliances, then made of gold and that were fixed to the tooth thanks to waxed linen threads (Jones, 2008).

The history of dental floss, like that of periodontics, is also linked to ancient civilizations. However, to find examples of dental floss similar to those we use today we must go back to the nineteenth century when a dentist in his New Orleans practice, Levi Spear Parmly, began to recommend his patients to use this set of fine filaments to deepen their buccal hygiene (Armitage, 2004).

This progress started with the development of modern toothpaste. It would begin almost immediately after Dr. Riggs defined gingivitis, known as ‘Riggs disease’ in those days.

Toothpaste has been since at least 500 BC, but innovations were happening. The first modern toothpaste began being mass-produced by Colgate in 1873 (Gum Disease, 2022.).

Toothbrushes fit a broad range of descriptions in those days. Manufacturers used everything from horse hairs to feathers, boar bristles to swine hair were used. The development of the modern toothbrush can be seen in a toothbrush made with the bone of cattle. It used metal staples to secure swine hair into the brush. The development of the plastic toothbrush wouldn't happen until 1938 (Sammons 2003).

In 1906, Fones taught his dental assistant cousin, Irene Newman, to instruct and treat his patients to maintain their mouths in a clean state. His customized educational program was presented publicly at the National Dental Association Meeting in Cleveland in July 1911. The first dental hygiene education program included these elements:

- Drawings and books for the study of dental anatomy
- Extracted teeth with penciled markings to be removed with orangewood sticks and wet pumice
- Observation using a hand mirror while Fones cleaned Newman's teeth
- Fones becoming Ms. Newman's patient and instructing her as he observed in the hand mirror (Kumar 2011).

All these procedures were repeated many times prior to treating patients in Fones' practice. Newman's first patients were children and she only polished teeth. Later, she began to scale teeth with instruments but was only permitted to remove gross deposits. Fones found that her services saved him a great deal of chair time, and as her skills improved she was able to further treat his patients (Patey, 2013).

Fones went on to establish the first school for dental hygienists in Bridgeport, Connecticut, in 1913. His school graduated hygienists for 3 years before colleges and universities began to train dental hygienists in 1916 (Eibasakis, 2023).

It is clear that periodontitis severely affects a high-risk group representing around 10–15% of the population, in whom the disease quickly progresses from chronic gingivitis to destructive periodontitis (Johnson, 1988).

Currently, there are two major forms of periodontitis: chronic and aggressive periodontitis (Armitage 2000). This differential risk for periodontitis is consistent with heritable elements of susceptibility, but direct evidence for a differential genetic contribution to periodontitis comes from several sources.

Many works of the literature report familial aggregation of periodontal diseases, but due to different terminology, classification systems, and lack of standardized methods of clinical examination, it is difficult to compare reports directly. Although periodontal disease nosology has changed many times over the timeframe of these reports, most familial reports for periodontitis are for early-onset forms now called aggressive periodontitis (Cohen and Goldman 1960, Butler 1969).

In chronic periodontitis, the phenotype or disease characteristics do not present significantly until the third decade of life, whereas, in the aggressive forms of periodontal disease, the presentation can occur in the first, second, third, and fourth decades. This variability

in presentation of significant signs of disease makes diagnosis difficult, not only in declaring if a patient suffers from the disease but also in detecting patients who do not suffer from the disease and differentiating between adult and aggressive forms of periodontitis. The problems associated with the clinical differentiation of periodontal disease are not uncommon in medical genetics, since similar problems arise in the study of other delayed-onset hereditary traits (Boughman et al., 1988).

The most recent classification we use dates from 2018 - Disease Classification for periodontal health: clinical gingival health on an intact periodontium; clinical gingival health on a reduced periodontium (Gehrig et al., 2019).

Gingivitis could be dental biofilm-induced, associated with dental biofilm alone, medicated by systemic or local risk factors, or drug-influenced gingival enlargement. Also, gingival diseases with nondental biofilm-induced is a genetic or developmental disorder, or caused by specific infections, or inflammatory and immune conditions. It could be produced by reactive processes, neoplasms, endocrine, nutritional, and metabolic diseases, traumatic lesions and gingival pigmentation.

The same classification states three types of periodontal diseases: *Necrotizing periodontal diseases* (necrotizing gingivitis, necrotizing periodontitis, necrotizing stomatitis), *Periodontitis* as manifestation of systemic diseases or *Periodontitis*.

Non-surgical therapy is the golden standard of periodontal therapy which consists of debridement with a combination of oral-hygiene instructions and patient motivation. It mainly focuses on the elimination and reduction of putative pathogens and shifting the microbial flora to a favourable environment to stabilize periodontal disease (Mordohai, et al., 2007). This phase aims to reduce and eliminate any gingival inflammation. During this stage dental plaque and calculus are eliminated, restoration from tooth decay and correction of defective restoration are taking place. It considers antimicrobial therapy, diet control, education of the patients, the control of iatrogenic factors, deep caries, hopeless teeth, preliminary scaling, temporary splinting, occlusal adjustment, minor orthodontic tooth movement and debridement (Mendes et al., 2010).

Phase II therapy - surgical phase - is required are because of periodontal pocket management in specific situations, irregular bony contours or deep craters, areas of suspected incomplete removal of local deposits, furcation involvements, distal areas of last molars with expected mucogingival junction problems, persistent inflammation, root coverage and removal of gingival enlargement. Surgical therapy may require a restorative phase, when defects need to be restored with removable or fixed through dental prosthesis, prosthodontics, or other restoration processes.

Each of these phases requires long time maintenance phase, by preservation of periodontal health. In this phase, patients are required to re-visit through a scheduled plan for maintenance care to prevent any re-occurrence of the disease (Graetz 2022).

The maintenance of the restorative treatment is determined by the periodontal health. Stage IV periodontitis is associated with an increased risk of tooth loss. Teeth with PPD $\geq$ 5 mm at the end of APT are at risk of periodontitis progression or tooth loss. Periodontal therapy should

follow restorative method as the resolution of gingival inflammation may result in the repositioning of teeth or in soft tissue and mucosal changes (Siow et al., 2023).

Given how many factors involved and given how little we know about what may be happening at any given moment, we can reasonably consider the influence of the multitude of determinants involved over a given period as being effectively random and constituting 'noise', some enhancing inflammation, some enhancing recovery, and some perhaps having no influence. While there may be noisy events happening within the biofilm, it is necessary to consider how the tissues are affected by this noise: the tissues respond to these stimuli by accumulating the effects of all the different components of noise (that is to say, the effects on the tissues of the positive, negative and neutral events are added together) (Dahlen et al., 2020).

Careful diagnosis, elimination of the causes and reduction of modifiable risk factors are paramount for successful prevention and treatment of periodontitis. Initial non-surgical periodontal therapy primarily consists of home care review and scaling and root planning. For residual sites with active periodontitis at periodontal re-evaluation, a contemporary regenerative or traditional resective surgical therapy can be utilised. Thereafter, periodontal maintenance therapy at a regular interval and long-term follow-ups are also crucial to the success of the treatment and long-term retention of teeth. The aim of this review is to provide current concepts of diagnosis, prevention and treatment of periodontitis. Both clinical and biological rationales will be discussed (Kaur 2023; TaeHyun Kwon et al., 2021).

Failure to do so often results in needless, inappropriate treatment, or just a wholesale extraction of teeth in favor of implants. Rest assured that when extraction occurs, little if any assessment of the causative agents ever crosses the clinician's inquisitiveness, as opposed to the cognizant pursuit of knowledge, and intuitive evaluations as seen and documented by our forefathers. This is the key to the dilemma and focus of this historical assessment and which raises a plea for the incorporation of meaningful historical data into contemporary thought and application (Bingham et al., 2023).

Treatment of recession defects is indicated for the prevention of root caries, reducing root hypersensitivity, enhancing esthetics, augmenting keratinized tissue, eliminating inconsistency of the gingival margin, and to enhance plaque control.

Periodontal regeneration occurs when epithelial cells are excluded from the graft site, and the defect is repopulated with periodontal ligament-derived mesenchymal stem cells (Tatakis et al., 2015).

Enamel matrix derivative (EMD, Emdogain) is a protein-rich gel extracted from porcine tooth buds which stimulates osteoblast proliferation. Studies have shown the use of EMD can stimulate the formation of new bone, cementum, and periodontal ligament on previously diseased root surfaces.

A recent review by the American Academy of Periodontology concluded that EMD is generally comparable with demineralized freeze-dried bone allograft and guided tissue regeneration (GTR) in inducing faster reepithelialization, wound closure, resolution of inflammation, and angiogenesis (accelerated new blood vessel formation) (Chambrone and Tatakis 2015).

Recombinant Growth Factors are genetically-engineered versions of human platelet-derived growth factors produced in the laboratory. They are identical in structure and action to the naturally-occurring signaling protein cells. Recombinant human platelet derived growth factor BB (rhPDGFBB) is often used in combination with xenografts and allografts in the treatment of intrabony defects. The use of rhPDGF-BB has been shown to result in improved periodontal clinical parameters of greater bone formation and decreased healing times when compared with the use of bone graft alone (Chambrone et al., 2018).

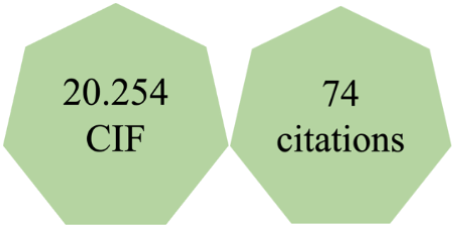
There are three basic surgical procedures used in the treatment of recession defects: free soft-tissue autografts, allografts and xenografts, free gingival graft, subepithelial connective tissue graft, pedicle soft-tissue autografts, coronally positioned flaps, rotational flap procedures, such as the laterally sliding flap and others or soft-tissue graft procedures in combination with regenerative procedures (Zuhr et al., 2013).

Although the future of periodontal therapy is bright, it is still of critical importance to have a preventive strategy to keep individuals healthy beforehand and the more promising research directions for a substantial regeneration seems to lie in biological mediators.

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

1. **Luchian, I.;** Goriuc, A.; Sandu, D.; Covasa, M. The Role of Matrix Metalloproteinases (MMP-8, MMP-9, MMP-13) in Periodontal and Peri-Implant Pathological Processes. *Int. J. Mol. Sci.* **2022**, *23*, 1806. https://doi.org/10.3390/ijms23031806
2. Sioustis, I.-A.; Martu, M.-A.; Aminov, L.; Pavel, M.; Cianga, P.; Kappenberg-Nitescu, D.C.; **Luchian, I.;** Solomon, S.M.; Martu, S. Salivary 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*, 1583. https://doi.org/10.3390/ijerph18041583
3. **Luchian, I.**; Moscalu, M.; Goriuc, A.; Nucci, L.; Tatarciuc, M.; Martu, I.; Covasa, M. Using Salivary MMP-9 to Successfully Quantify Periodontal Inflammation during Orthodontic Treatment. *J. Clin. Med.* **2021**, *10*, 379. https://doi.org/10.3390/jcm10030379
4. Ursu, R.G.; Iancu, L.S.; Porumb-Andrese, E.; Damian, C.; Cobzaru, R.G.; Nichitean, G.; Ripa, C.; Sandu, D.; **Luchian, I**. Host mRNA Analysis of Periodontal Disease Patients Positive for *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia*. *Int. J. Mol. Sci.* **2022**, *23*, 9915. https://doi.org/10.3390/ijms23179915
5. Nanu, S; Goriuc, A; Bejan, O; Vata, I; Martu, M.-A.; Testa, S; Sandu, D.; Butnaru, O; **Luchian, I**. A Comparative Study between Periodontal Status before and after Orthodontic Treatment. *Rom. J. Oral Rehab.* **2022**, 14,4.

These articles gathered the following scientometric parameters according to Clarivate:



1.6. Salivary Metalloproteinase-8 and Metalloproteinase-9 quantification in periodontal disease

1.6.1. Introduction

Severe periodontitis, a widely spread severe inflammatory disease leading to the destruction of the supporting tissues of the teeth, affects approximately 11% of the global population (Tannure et al., 2012). It is estimated that this condition causes losses of billions of dollars globally each year, both through high direct costs of treatment and indirectly by affecting the condition of patients (Dezerega et al., 2012). However, periodontitis treatment is relatively simple and cost-effective if diagnosed in the early stages. Quite challenging, periodontitis is usually asymptomatic, meaning that a person with the disease is unaware of it, and its traditional diagnosis requires specialist doctors and equipment that is often not available, especially in poor environments (Grant et al., 2013; Kim et al., 2013).

The development of microorganisms at the dental level (the main cause of periodontal damage) involves a wide range of macromolecules, including many proteins produced by different cells. These proteins include matrix metalloproteinases (MMPs) involved in the degradation of the extracellular matrix and whose functions are modulated by tissue metalloproteinase inhibitors called TIMPs. MMPs (or matrixins) are an important family of calcium-dependent zinc-containing endopeptidases; they are able to degrade not only extracellular matrix proteins but also non-matrix proteins, including cytokines (Zhang et al., 2018).

Degradation of extracellular matrix proteins (ECMs) by proteinases is a key feature of periodontal disease and can be derived from both microorganisms in dental plaque and from cellular sources (Sorsa et al., 1995). Previous studies have shown that proteases derived from both microbial and cellular sources can contribute to the activation cascade of MMPs, leading to periodontal destruction (Yakob et al., 2013). Moreover, microbial proteases stimulate proteolytic activators of latent human pro-MMPs, which can increase their secretion by gingival resident cells, causing the degradation of ECM collagen.

MMPs are involved in multiple pathological processes in the body, including oncological pathology (Gonzalez-Avila et al., 2019). Some metalloproteinases are involved in metastases, others are associated with the level of tumor aggression, and some of them are either used as markers for the diagnosis of certainty or for prognosis determination. For example, MMP-2 is involved in hard dental tissues, favouring a dental tissue much more sensitive to karyogenic acid

attacks, by degrading the enamel proteins such as amelogenin (Checchi et al., 2020). Increased activity of MMP-2 and MMP-9 has been observed in apical lesions following pulpal necrosis, which may demonstrate their involvement in the development of apical periodontitis. Given that numerous studies have shown an increased expression of MMP-9 and MMP-8 activity in patients with periodontal disease, they have been proposed as valid indicators of the disease (Khuda et al., 2021). For example, in patients subject to orthodontic treatment, high levels of MMPs, especially MMP-2 and MMP-9, have been found at the sites of tension and compression of the teeth undergoing orthodontic treatment (Luchian et al., 2021).

- **Matrix Metalloproteinases and Their Physiopathological Involvement**

The MMPs were first described as early as 1949 with the discovery of depolymerizing enzymes involved in connective tissue growth (Gersh and Catchpole 1949). However, the research into what is currently known as the family of matrix metalloproteinases began in 1962 when Woesnner, followed by Gross and Lapiere, discovered and characterized an enzyme with collagenolytic activity in the amphibian tissues (Birkedalhansen 1988). It was only in 1980 that Harris et al. proposed using the name MMPs for this group of collagenases/gelatinases. The past 60 years have seen remarkable progress in studying the biological functions of MMPs and their involvement in numerous biological processes such as tissue repair and remodelling, cellular differentiation, embryogenesis, morphogenesis, cell proliferation, apoptosis, would healing, and reproduction, to name a few (Laronha and Caldeira 2020). Not surprisingly, the deregulation of MMPs activities leads to several pathological processes and diseases such as periodontal diseases, arthritis, cancer, neurodegenerative disorders, cirrhosis, and cardiovascular abnormalities.

MMPs (or matrix metalloproteinases) are major enzymes involved in extracellular matrix remodelling; they can also act intracellularly, are capable of activating growth factors in their proximity, cell surface receptors, and adhesion molecules (Dom et al., 2016), and are classified based on the specificity of the substrate. Despite the fact that they largely represent a sequence of similar structures, there are still differences in the substrate specificity (Hannas et al., 2007) represented by degraded matrix proteins (Table I.2.1.). Currently, there are 28 members in this family, with 25 of them present in humans (Gonzalez-Avila et al., 2019; Checchi et al., 2020). They are described and classified into five sub-families based on their function and structure: collagenases, gelatinases, stromelizines, membrane matrix metalloproteinases (MT-MMPs), and other MMPs (Laronha and Caldeira 2020). Some of the domains in the structure of MMPs are indispensable for the intracellular transport of secreted enzymes to the cell membrane, which are finally eliminated after the protease is secreted. MMPs also have a domain with latent enzyme capacity.

The family of metalloproteinases with a particularly important role in numerous physiological and pathological processes at the tissue level is, in fact, represented by a group of enzymes that participate in cleaving the components of the extracellular matrix. Given their role in tissues, they are represented by collagenases, including MMP-1, MMP- 8, MMP-13, and MMP-18, which can degrade interstitial collagen I, II, III, resulting in degraded collagen or gelatin. MMP-1, which is synthesized by macrophages, fibroblasts, and dentritic cells, is

involved in promoting cell survival. On the other hand, MMP-8, secreted by neutrophils, has antitumor action and anti-invasive properties due to its role in regulating hormone receptors (Sapna, et al., 2014).

Gelatinase A (MMP-2) and gelatinase B (MMP-9) are two proteins in this family that are responsible, among other things, for the degradation of type IV collagen in the basement membrane (Hannas et al., 2007). Gelatinase A (MMP-2), with a molecular weight of 72 kDa, is involved in the degradation of type I, II, and III collagen and, under normal conditions, is expressed by stromal cells in most tissues (hematopoietic, endothelial, dendritic cells, fibroblasts, mast cells, and macrophages). Gelatinase B (MMP-9) has a molecular weight of 92 kDa, is found in very small amounts in normal tissues, and is secreted by dendritic, hematopoietic, macrophage, neutrophil, fibroblast, and lymphocyte cells (Nazir et al., 2020).

Table I.2.1. The main metalloproteinases: name, substrates, and the main diseases in which they are involved.

Type of MMP	Name	Substrate	Production	Physiological Function	Associated Disease's	References
MMP-1	Collagenase1/ Interstitial Collage-nase/Fibroblast Collagenase	Collagen I, II, III, VII, VIII, X, XI, Gelatin, Fibronectin, Aggrecan, Entactin, Tenascin, Ovostatin, Casein	Fibroblast, Keratinocytes, Endothelial cells, Macrophages, Osteoblast, Chondrocytes, Platelet	Wound healing, re-epithelialization, cell proliferation, Keratinocyte migration	Periodontitis Rheumatoid arthritis, Atherosclerosis, Fibrosis, Autoimmune disease, Cancer	(Laronha and Caldeira, 2020; Verma and Hansch, 2007; Li et al., 2016)
MMP-2	Gelatinase A/72-kDa type IV collagenase	Collagen, Elastin, Endothelin, Fibroblast growth factor, MMP-9, MMP-13, Plasminogen, and TGF-β,	Cardiomyocytes, Fibroblasts, and Myofibroblasts.	Neovascularization, Angiogenesis, Promoting and inhibiting Inflammation,	Cancer, asthma, lung diseases,	(Cui, Hu and Khalil, 2017; Yabluchanski y et al., 2013)
MMP-8	Collagenase2/ Neutrophil Collagenase	Collagen I, II, III, Fibronectin, Aggrecan, Ovostatin	Chondrocytes, Endothelial cell, Macrophages, Smooth muscle cell	Periodontal tissue turnover, Anti-inflammatory activity, Wound healing	Periodontitis, Rheumatoid arthritis, Asthma, Cancer	(Cui, Hu and Khalil, 2017; Ye, 2015; Nagase, Visse and Murphy, 2006)
MMP-9	Gelatinase B/ 92-kDa type IV collagenase	Gelatin, Type V collagen, Laminin, Fibronectin	Neutrophils, Eosinophils, Epithelial cells	Wound healing, Embryo implantation, Neovascularization, immune cells function, tissue remodeling	Arthritis, Metastasis, Pulmonary disease, Infections, Cardiovascular disease, Periodontal disease	(Yabluchanski y et al., 2013; Ye, 2015)
MMP-12	Macrophage elastase	Elastin, Laminin, Fibronectin, Vitronectin, Type IV collagen	Endothelial cells, Neutrophils, Fibroblasts, T-cells, Myocytes, Macrophages,	degrade extracellular matrix component	Emphysema, Arthritis, Cancer, Periodontal disease	(Gonzalez-Avila et al., 2019; Molet et al., 2005)
MMP-13	Collagenase 3	Collagen I, II, III, IV, IX, X, XIV, Fibronectin, Laminin, Gelatin, Aggrecan, Plasminogen, Osteonectin	Epithelial cell, Neuronal cell, Connective tissue (Cartilage and Bone)	Osteoclastic activation, Anti-inflammatory activity	Periodontitis, Osteoarthritis, Liver fibrosis, Cancer	(Cui, Hu and Khalil, 2017; Ye, 2015; Yamamoto et al., 2016, Chow and Chin, 2020)

MMP-3, MMP-10, and MMP-11 are also called stromelizines and are involved in the digestion of certain molecules in the extracellular matrix and the basement membrane. MMP-3 indirectly modulates cell migration and is secreted by fibroblasts, lymphocytes, endothelial, and dendritic cells. MMP-11 is located mainly in the adipose tissue in the proximity of a tumor, being correlated with adipogenesis processes. MMP-11 is also a negative modulator of pre-adipocyte differentiation and reverses the differentiation of mature adipocytes, which leads to the peritumoral accumulation of fibroblast-like cells, thus favoring tumor progression (Van Dyke and Sheilesh, 2005). Matrilizines MMP-7 and MMP-26 are the simplest proteins of this family from a structural point of view because they do not present a domain. They act on cell surface molecules and are expressed mainly by tumor cells of epithelial origin. MMP-7 is secreted by macrophages, endothelial cells, and osteoclasts, thus being involved in inflammatory processes, cell invasion, and angiogenesis (Verma and Hansch, 2007). Membrane matrix metalloproteinases are part of the basement membrane and modulate the proteolytic activity of other MMPs. They can be divided into transmembrane proteins that bind through a hydrophobic region, such as MMP-14, MMP-15, MMP-16, MMP-24, and proteins with a glycolphosphatidylinositol group (GPI), such as MMP-17 and MMP-25.

The most studied matrix metalloproteinase is MMP-14, which is expressed by fibroblasts, macrophages, endothelial cells, and hematopoietic cells. They are involved in cell growth and in stimulating adipogenesis and angiogenesis. In addition to metalloproteinases, which are grouped according to their structure and role, there are the MMPs, themselves representatives of a family. One such example is MMP-12, a metalloelastase that is synthesized by macrophages with elastin as a substrate and whose migratory capacity is affected (Franco et al., 2017). Another metalloproteinase called enamelizine or MMP-20 has, as substrate members of the amelogenin family, extracellular matrix proteins. MMP-28 is expressed in keratinocytes and plays a very important role in hemostasis and wound healing. In addition to all these MMPs with known and well-defined functions, there is also MMP-22, whose functions are not well known, and MMP-23, which is predominantly expressed in reproductive tissue and which does not present the hemopexin domain (Nazir et al., 2020). (Figure I.2.1.).

The activity of metalloproteinases is modulated by tissue inhibitors of metalloproteinases-TIMPs, which comprise four types. An example of such a molecule is TIMP-1, secreted by neutrophils, lymphocytes, and mast cells that can inhibit most MMPs, except for membrane-type metalloproteinase MT1-MMP and MMP-2 (Van Dyke and Sheilesh, 2005).

TIMP-2 is secreted by hematopoietic, dendritic, and endothelial cells and inhibits the activity of most MMPs, except MMP-9. TIMP-3 inhibits the activity of MMP-1, -2, -3, -9, and the membrane-type metalloenzyme MT1-MMP (Li et al., 2016). All these are also synthesized by macrophages and fibroblasts. As previously mentioned, matrix metalloproteinases play a very important role in various physiological and pathological processes, being responsible for the degradation of extracellular matrix components such as fibers (collagen, elastin, laminin, and fibronectin) and the degradation of proteoglycans and polysaccharides (Verma and Hansch, 2007; Checchi et al., 2020).

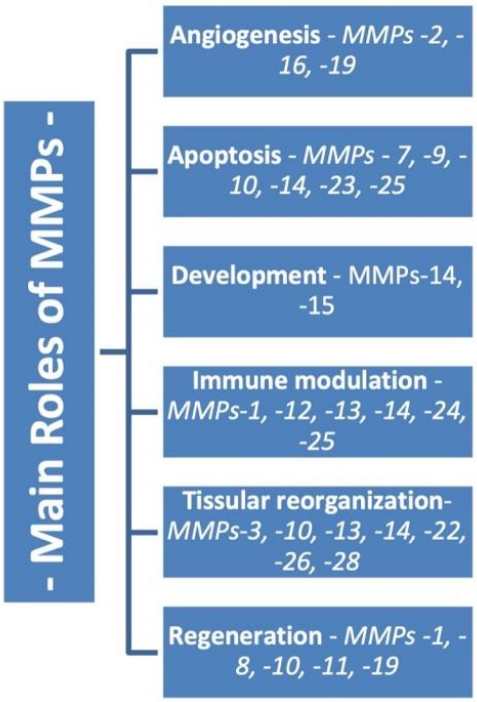


Figure I.2.1. The main functions of matrix metalloproteinases

These fibers are mostly collagen-containing glycoproteins, which are considered the main protein in the extracellular matrix. There is a common structure for most members of the metalloproteinase family that comprises a pro-peptide consisting of 80 amino acids with a variable-length N-terminal signaling peptide end, a catalytic domain containing a Zinc ion (170 amino acids), the hemopexin domain involved in collagen degradation (200 amino acids), and a region represented by a variable-length binding peptide, linking the catalytic domain to the hemopexin domain (Klein and Bischoff, 2011) (Figure I.2.2.).

Despite the many similarities between the members of the MMP family, there are also differences. For example, MMP-7, -23, and -26 do not have the binding peptide or the hemopexin region, while MMP-14, -15, -16, and -24 also have a C-terminus end. MMP-23 exhibits a cysteine arrangement and an Ig-like domain instead of the binding region and the hemopexin domain (Sela-Passwell et al., 2010).

In general, the activity of MMPs is closely linked to the existence of a balance between the levels of MMPs and TIMPs, and any alteration of this balance can lead to the progression of the disease/inflammation (Verstappen and Von den Hoff, 2006). Four endogenous inhibitors present in humans, called TIMP-1, TIMP-2, TIMP-3, and TIMP-4, are involved in modulating the activity of MMPs. They have a 40% similar structure, with the difference being represented by the specificity of the substrate. These inhibitors have a role in restoring the components of the extracellular matrix, tissue, and cell remodelling, all of which are mediated by the MMPs themselves (Verstappen and Von den Hoff, 2006; Franco et al., 2017).

TIMPs are inhibitors with high specificity due to the chelating action of Zn2+ in the structure of the catalytic domain of MMPs, thus regulating the enzymatic activity of MMP. This complex (MMP-TIMP) is not affected by denaturation through heating or proteolysis (Visse and Nagase, 2003). MMPs are involved in various biological processes, including cell proliferation, tissue repair, differentiation, migration, healing, morphogenesis, angiogenesis, and apoptosis (Table I.2.1.).

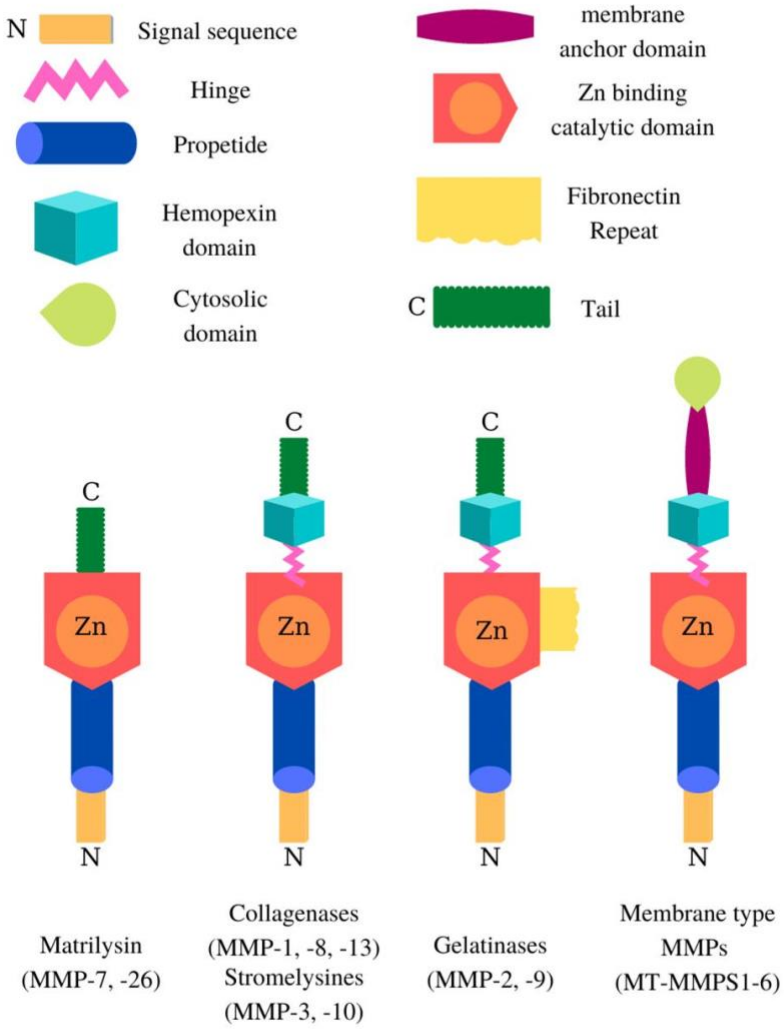


Figure I.2.2. Modular organization of the MMP domain.

TIMPs are inhibitors with high specificity due to the chelating action of Zn2+ in the structure of the catalytic domain of MMPs, thus regulating the enzymatic activity of MMP. This complex (MMP-TIMP) is not affected by denaturation through heating or proteolysis (Visse and Nagase, 2003). MMPs are involved in various biological processes, including cell proliferation, tissue repair, differentiation, migration, healing, morphogenesis, angiogenesis, and apoptosis (Table I.2.1.).

An imbalance in the regulation of MMPs activity can lead to tissue destruction, fibrosis, and degradation of the extracellular matrix, which represent various stages of the disease progression, including periodontal disease (Laronha and Caldeira 2020). The MMPs degrade the components of the extracellular matrix, the basement membrane, and certain enzymes (SERPINs—serine protease inhibitors), which have a great impact on cytokines, osteoclast activation, tissue regeneration, and loss of connective tissue attachment.

The fibroblasts that are part of the desmodontium and also the gingival cells synthesize collagenases such as MMP-1, -8, and -13. On the other hand, MMP-2 and MMP-9 are mainly synthesized by neutrophils and macrophages and are involved in MMPs-mediated destructive periodontal disease. Increased amounts of these MMPs are released from epithelial cells, which can influence apical migration and lateral extension of the junctional epithelium. Finally, these processes lead to the loss of connective tissue attachment (Charles et al., 2014).

Although many studies have shown that several MMPs contribute to the progression of periodontal disease, such as MMP-2,-7, and -14, the most reported MMPs that are responsible for periodontopathy are collagenases MMP-1, -8, and -13. This group of collagenases has the ability to degrade almost all types of collagenous and non-collagenous proteins in the extracellular matrix. Previous studies have shown that MMP-1 and MMP-8, in addition to being intensively involved in periodontal pathology, are also strongly correlated with cardiovascular pathology and diabetes (Lahdentausta et al., 2018). Table 1 highlights the substrate, production, and physiological functions and the associations with other collagenase-induced diseases, such as MMP-1, -8, and -13.

- **The Relationship between MMPs and Periodontal Disease**

Treponema denticola, considered a periodontal pathogen, secretes certain proteases that activate pro-MMP-2, released in its inactive form by the periodontal ligament cells. MMP-2 will thus produce a destructive phenotype, causing fibronectin fragmentation, induction of apoptosis, or suppression of osteoblast differentiation. Several studies have demonstrated that Porphyromonas gingivalis may, in addition to gingival pain, activate the secretion of MMP-2 (Leppilahti et al., 2014). It also increases the migration of monocytes by activating the expression of MMP-9, which will indirectly lead to tissue destruction. Since, compared to MMP-2, MMP-9 has been shown to exert an increased activity and is largely present in periodontal patients, MMP-9 could be considered a predictor of disease activity (Yakob et al., 2013). Indeed, a decrease in the levels of MMP-1, -8, -9, -12, and -13 in the crevicular fluid has been observed following the treatment of aggressive periodontitis by descaling and surface enhancing, alongside antibiotic treatment. This demonstrates that the levels of MMPs are in a dynamic balance with the

state of hygiene and health of periodontal tissues. Therefore, the presence of these proteases may facilitate the installation of a destructive microenvironment in the periodontium, which would determine the manifestation of the periodontal disease (Verma and Hansch, 2007, Klein and Bischoff, 2011).

MMP-1, -8, and -13 have been detected in the peri-implant sulcular fluid, and this is associated with increased activity of annual vertical bone loss. In these cases, MMP-8 can be considered a possible marker for progressive bone loss in implants (Gursoy et al., 2013; Balli et al., 2016).

The levels of matrix metalloproteinases from the gingival crevicular fluid of patients with periodontal disease have also been studied from a diagnostic and prognostic point of view. For example, a cross-sectional study by Ramseier et al. found that salivary concentrations of MMP-8, -9, and orthopantomograms, combined with the presence of bacteria, can predict periodontal disease (Ramseier et al., 2009).

GCF levels of MMP-9 and -13 have been suggested as useful biomarkers for the progression of periodontitis in patients with moderate chronic periodontitis who have active sites and who have been observed for 2 months. These findings are in line with other cross sectional studies showing that GCF levels of MMP-8 and -9 are correlated with disease activity in patients with chronic periodontitis (Kraft-Neumarker et al., 2012; Sorsa et al., 2020).

Interesting data also emerged from a clinical study with 28 patients with chronic periodontitis and 22 controls (Lenglet et al., 2013; Juurikka et al., 2019). The authors reported higher plasma levels of MMP-3, -8, -9 in patients with chronic periodontitis compared with controls, which decreased significantly 3 months after non-surgical periodontal treatment. Kinane et al. also reported that the GCF levels of MMP-8 decreased significantly 3 months after non-surgical periodontal therapy in 20 patients with chronic periodontitis (Kinane, 2001).

Persistent increase of MMP-8 in GCF samples is considered a high risk of poor response to periodontal therapy (Yakob et al., 2012). Moreover, significant positive correlations were detected between MMPs-8 and -9 activities in GCF and periodontal disease severity, along with negative correlations with TIMP-1 and -2 levels. Therefore, a chairside MMP-8 test would be advisable to effectively differentiate clinically healthy sites and gingivitis from chronic periodontitis and also to effectively monitor the treatment of patients with chronic periodontitis (Honibald et al., 2012).

When it comes to bone resorption, MMP-9 is probably the most important proteinase involved in this process because osteoclasts express this enzyme at an extremely high level. However, there are conflicting reports about the specific role of MMP-9 in bone resorption. For example, some studies have suggested that MMPs have, at best, a very small contribution to osteoclast bone resorption activity and that the selective MMP-9 inhibitor, TIMP-1, did not show a significant inhibitory effect on osteoclastic bone resorption (Rathnayake et al., 2017), while other studies have shown that MMP-9 may play a key role in bone resorption caused by osteoclasts and that patients with MMP-9 genotypes, in association with their soluble protein, may have an increased risk of developing chronic periodontitis (Yakob et al., 2012; Leppilahti et al., 2014). Application of orthodontic treatment can impact levels of MMPs. For example,

orthodontic forces on a tooth can generate tension and compression in the periodontal ligament, which affect there modelling of the alveolar bone and gingival tissue and are associated with high levels of MMP-1, -2 -8, and -9.

Chemical inhibition of MMP-9 reduces orthodontic dental movements. Gingival hypertrophy without signs of inflammation can be caused by the reaction of the gingival tissue to the mechanical stress induced by orthodontic forces, triggering the activity of MMP-9, which makes it a good marker in the gingival fluid and gingival tissue in this situation. MMP-9 levels are higher in chronic gingivitis but lower than in the presence of active periodontal disease (Ajmera et al., 2016; Hamodat and Taha, 2020).

- **Matrix Metalloproteinase 8 (Collagenase 2)**

General Aspects. The name collagenase denotes an enzyme capable of splitting tri-helical collagen. A collagenase isolated from human polymorphonuclear leukocytes (PMNLs) was first described in 1968 and was called PMNL-collagenase or neutrophil collagenase. Part of the extracellular matrix neutrophil collagenase is also called matrix metalloproteinase 8 (MMP-8) (Sorsa et al., 2016). At first, it was thought that this collagenase is expressed only in neutrophilic leukocytes, but its enzyme and messenger RNA were also found in cells such as normal human joint chondrocytes, mononuclear fibroblasts, human endothelial cells, and human odontoblasts in bronchial epithelial cells (Hannas et al., 2007), and it is the major collagenase in human dentin.

It is expressed during early development in neuronal crest cells and adult melanoma cells but has also been found in cells in oral squamous cell carcinoma. Following the discovery of a third form of human collagenase (MMP-13), neutrophil collagenase became commonly referred to as collagenase 2 (Sapna et al., 2014). Neutrophil collagenase is stored intracellularly as a latent proenzyme in specific granules of polymorphonuclear leukocytes. Procollagenase activation occurs in the extracellular space after secretion and can be stimulated by stromelysin, trypsin-2, cathepsin G, or other mediators. The activated enzyme is able to split tri-helical collagen type I, II, and III and has several proteolytic properties, including hydrolysis of natural substrates such as gelatinous peptides, fibronectin, proteoglycans, fibrinogen, and aggrecan cartilage, as well as serpine inhibitors such as human C1 inhibitor or α1-proteinase, β-casein, and human chemokines. The enzymatic activity of MMP-8 is inhibited by TIMPS and α 2-macroglobulin (Kiili et al., 2002).

The gene for human neutrophil collagenase is located on the long arm of chromosome 11. In addition to their main phagocytosis function, human PMNLs have a high capacity for infiltration into the connective tissue. This is often associated with a defect in the extracellular matrix, especially during pathological processes such as inflammation from rheumatoid arthritis or osteoarthritis but also from periodontal disease, and this is initiated by collagenase in human neutrophils. The MMP-8 expression is stimulated by IL-1β and inhibited by insulin-like growth factor 1 (Sbardella et al., 2012; Sorsa et al., 2016). The enzyme stored in specific granules is released from neutrophils as latent procollagenase under the action of various stimuli, such as interleukin 1 and 8, tumor necrosis factor α (TNF α), chemotactic formylpeptides, human

anaphylatoxin C5a, fibrinogen- and fibrin-derived products, granulocyte-macrophage colony-stimulating factor (GMCSF), and calcium ionophore A23187.

Several secreted forms have been described (Stawowczyk et al., 2017). The mesenchymal cells of the dentin and pulp express and activate MMP-8, which is inhibited by TGF-beta 1 (Shintani et al., 2017). The precise pathway of the in vivo activation after secretion remains unclear, although various agents have been reported to initiate the in vitro activation according to the "cysteine switch" activation mechanism. The activation may also be accomplished through autocatalysis by compounds containing mercury, oxygen radicals, hydrogen peroxide or hypochlorite, or sodium thialate (Siwik, Pagano and Colucci, 2001; Van Lint et al., 2005).

MMP-8 in Periodontal Diseases. Because type I collagen is the major component of the periodontal extracellular matrix, special attention has been paid to the role of collagenases, especially MMP-8 and MMP- 13 and gelatinases, MMP-2 and MMP-9, in periodontal diseases. In fact, MMP-8 (or collagenase 2) has been referred to as one of the most promising biomarkers for periodontitis in oral fluids (Verstappen and Von den Hoff 2006). Several MMPs have been detected in various enzymatic forms in the gingival tissue, gingival crevicular fluid (GFC), and saliva, with the main metalloproteinases involved in the destruction of dental tissue being MMP-8, MMP-13, and MMP-9 (Charles et al., 2014; Ye, 2015).

MMP-8 and MMP-9 are the most abundant MMPs in periodontal tissues, and their level reflects the severity of the disease and its progression and response to treatment. They are secreted due to the infiltration of polymorphonuclear leukocytes and also macrophages, plasma, and residual cells such as fibroblasts, endothelial cells, keratinocytes, and bone cells (Li et al., 2012).

Proteolytic cascades can lead to extensive destruction of the periodontal tissue due to the activation of MMPs and could be an interesting target for diagnosis and therapy. For example, MMP-13 is able to induce proMMP-9 activation and automatic activation of MMP-13 by in vitro auto-proteolysis. MMP-14 can activate MMP-8 and -13 in vitro, as well as MMP-2, and has been correlated in vivo with MMP-13 activation in periodontal sites (Cui et al., 2017).

Overall, these mechanisms could increase the destruction of periodontal tissues and could lead to the consequent progression of chronic periodontitis. In addition, nonproteolytic oxidative activation of MMP appears to be essential in periodontal inflammation. Reactive oxygen species (ROS) are cable of activating key MMPs in periodontal tissues through direct enzymatic oxidation but also through indirect mechanisms.

Neutrophil degranulation is stimulated by periodontopathogenic bacteria, cytokines, and prostaglandins, leading to the release of myeloperoxidase (MPO) from the primary granules (Saari et al., 1990).

An important biological function of MMP-8 in the periodontium is to facilitate the migration of leukocytes, especially neutrophil granulocytes, from the circulation to the periodontal sulcus by the cleavage of collagen and other components of the extracellular matrix (Luo et al., 2017). In addition, after the onset of inflammation, other cell types (e.g., fibroblasts) may also express MMP-8 (Verstappen and Von den Hoff, 2006). Increased expression, release, and activation of uncontrolled MMP-8, along with other MMPs and proteinases, are thought to

induce the inflammation associated with tissue destruction in periodontal disease and also other inflammatory diseases (Charles et al., 2014). The activation of MMP-8 is facilitated by other MMPs and host proteases and increased oxidative stress caused mainly by neutrophil-released myeloperoxidase (MPO).

Bacterial-derived proteases, such as Porphyromonas gingivalis (gingipain) and Treponema denticola, can also activate MMPs (Ramseier et al., 2009). MMP-8 is currently considered one of the most promising biomarkers used for the anticipation, diagnosis, prognosis of treatment, and classification of periodontal disease (Sorsa et al., 2020).

Several studies have reported significant increases in MMP-8 levels in subjects with chronic periodontitis and periodontitis associated with diabetes (Sapna, Gokul and Bagri-Manjrekar, 2014). For example, increased MMP-8 levels in GCF were reported due to the presence of periodontal pathogens such as T. denticola and T. forsythia, which represents a cascade of host responses induced by these organisms (Yakob et al., 2013). Effective periodontal treatment and MMP inhibitory adjuvant drugs have been shown to have an inhibitory effect in the progression of periodontal disease by reducing the level of MMP-8 in GCF and saliva (Sorsa et al., 1995). MMP-8 activity was also found to be altered in various organs and body fluids in smokers (Heikkinen et al., 2010) since tobacco induces degranulation in neutrophils and the growth of proinflammatory mediators that can influence the expression of MMP-8 in the periodontal environment of smokers. However, not all studies were successful in associating these levels of MMP-8 with smoking and the risk of periodontal disease (Liu et al., 2006; Honibald et al., 2012).

Several available collagenase inhibitors have been approved by the US Food and Drug Administration (USDA) and are analogs of tetracycline and doxycycline hyclate (Luizon and Belo, 2012). As such, subantimicrobial doses of doxycycline (SDD) have been widely accepted as an important adjuvant therapy in the treatment of periodontitis, and several studies have already shown its effectiveness.

- **Matrix Metalloproteinase 9 (Gelatinase B)**

General Aspects. MMP-9 is a proteolytic enzyme that decomposes type IV collagen, which is the basic structural component of the basement membrane. MMP-9-1562 C/T SNP, located on chromosome 20q11.2-q13.1, is a metalloproteinase investigated for its association with an increased risk of developing cancer, emphysema, and other diseases. Based on the evidence from previous studies, the suggested mechanism behind this association could be that MMP- 9 expression is primarily controlled at the transcriptional level, where the MMP-9 gene promoter responds to various stimuli such as cytokines and growth factors (Ajmera et al., 2016).

Moreover, the T allele of this variant can eliminate a binding site for a transcription repressor, altering the activity of the MMP-9 promoter, causing increased MMP-9 expression. In addition, changing the C-to-T site at position 1562 may alter nuclear protein binding in this region, resulting in increased macrophage transcriptional activity.

MMP-9 (or the gelatinase B) participates in the breakdown of various proteins from the connective tissue, including collagen type IV, V, and XI, proteoglycans, and elastin and is

abundantly expressed in chronic periodontitis (CP) (Bildt et al., 2008). Various cell lines, such as polymorphonuclear leukocytes, macrophages, keratinocytes, fibroblasts, osteoclasts, eosinophils, and neutrophils, have been linked to the expression of the MMP-9 gene, located on chromosome 20q11.2-13.1.

Genetic variations in the promoter region of the MMP-9 gene may have an effect on the transcription and synthesis of its proteins, which may influence the degradation of connective tissue of the protein and thus contribute to genetic susceptibility to periodontal disease (Checchi et al., 2020). Notwithstanding several studies examining the association of these polymorphisms with CP susceptibility and/or disease severity, the results thus far indicate a high degree of variability (Luizon and Belo, 2012).

MMP-9 and its relationship with periodontal disease

Among the MMP isoforms, MMP-9 has been validated in various preclinical models as one of the most common mediators present in the stages of inflammation progression in patients with periodontitis. MMP-9 has been shown to be released by vascular macrophages upon exposure to pathogenic bacteria or during the host response and has been validated as a subclinical marker of vascular homeostasis (Sapna et al., 2014; Laronha and Caldeira, 2020). MMP-9 has been found to regulate some mediators during the early stages of inflammation, including IL-1, -6, and -8, and prostaglandins (Li et al., 2016; Franco et al., 2017).

Preliminary evidence has shown that MMP-9 expression is associated with damage to periodontal tissue during the active stages of periodontitis (Gursoy et al., 2013). In this regard, some studies have shown that MMP-9 is increased in the gingival crevicular fluid during the initial phase of periodontitis, having a key role in the neoangiogenesis associated with the host response to periodontal pathogens (Soder et al., 2009).

The main source of MMP-9 is the polymorphonuclear neutrophils, with high levels being expressed in inflamed junctional and gingival epithelial cells in advanced periodontitis (Yabluchanskiy et al., 2013). Thus, high MMP-9 levels may accurately reflect the condition of patients with periodontitis and can be a useful biomarker for diagnosis (de Araujo et al., 2017).

During periodontitis, it has been hypothesized that MMP-9, together with CRP (Creactive protein), may inhibit the synthesis of nitric oxide (NO), which, in turn, may adversely affect the endothelium and the arterial vascular tone and eventually lead to endothelial dysfunction and an increased risk of cardiovascular disease (Holla et al., 2006). Indeed, several studies showed a reciprocal relationship between endothelial damage due to NO release and serum levels of MMP-9. In addition, periodontal disease has been closely linked to high levels of NO, indicating a direct correlation between serum NO, MMP-9, and periodontitis. During the development of periodontal disease, the regulation of serum NO levels is related to the immune response of the host, which occurs as a result of infection with pathogenic periodontal bacteria (Schenkein and Loos, 2013).

Notwithstanding these findings, there is currently no full consensus regarding the effects of NO and direct oxidative stress on periodontal tissues. Indeed, some studies have shown high levels of NO and MMP in patients with periodontitis in the active phase of the disease, while other evidence has shown low levels of NO and some classes of MMPs during the development

of periodontal disease (Diaz et al., 2020). A possible explanation for the results of this study comes from other evidence showing that MMP-9-mediated immune response is associated with the presence of heat shock proteins released during periodontitis, exerting a specific action on T lymphocytes (Siwik et al., 2001). In this regard, recent studies have validated MMP-9 as an essential modulator of host defense mechanisms during the initial immune phase, which, in turn, can trigger a cascade of events involving the entire host defense mechanism, the endothelial homeostasis, leading to an increased risk of developing periodontal disease (Ramseier et al., 2009; Leppilahti et al., 2014). Thus, targeted therapy focused on inhibiting MMP activity could be an ideal therapeutic option in the treatment of periodontal disease, in addition to scaling, root surfacing, and bone surgery.

- **Matrix Metalloproteinase 13 (Collagenase 3)**

General Aspects. In 1994, a new human matrix metalloproteinase with the structural features of a collagenase was identified and named collagenase 3 or metalloproteinase-13 (MMP-13). MMP-13 is a proteolytic enzyme belonging to a large family of endopeptidases responsible for extracellular matrix degradation, and it is characterized by the binding of zinc to their catalytic site (Leeman et al., 2002). The MMP-13 gene is located on chromosome 11q22.3, like other MMP genes. Thus, MMP-13 is a metalloproteinase secreted in the form of a proenzyme and includes (made up of) 471 amino acids. The activated form of MMP-13 has a catalytic domain and a domain similar to the hemopexin responsible for the degradation properties of MMP-13.

Although the catalytic domain of MMP-13 itself can even degrade collagen, it is not as effective as the domain of hemopexin. MMP-13 is a very important metalloproteinase during skeletal growth and long bone maturation, as the MMP-13-mediated degradation of pre-existing extracellular matrix proteins has proved to be a necessary and important step in bone development prior to neoangiogenesis and mineralization. MMP-13 is oversynthesized in various pathological conditions, being involved in the degradation of collagen, aggrecan, fibronectin, and tenascin as well as other extracellular matrix proteins. Thus MMP-13 has an essential role in the progression of human carcinoma and metastatic processes, the development of acute articular rheumatism, and osteoarthritis (Malemud, 2017).

As such, MMP-13 is overexpressed in the cartilaginous tissues of patients with osteoarthritis, and an increased level of MMP-13 in chondrocytes may be an initial mechanism in the development of osteoarthritis (Cui et al., 2017). Although, initially, MMP-13 was thought to be revealed in the connective tissue, especially in developing cartilage and bone, the epithelial tissue and neuronal cells also contain MMP-13. MMP-13 demonstrates versatility in the use of its substrate. In addition to being very active on type II collagen, MMP-13 breaks down other substrates, mainly extracellular matrix macromolecules, but also molecules such as the connective tissue growth factor (CTGF) and fibrinogen. MMP-13 is controlled at several levels: by controlling expression/ synthesis but also by activating/inhibiting the active form of the enzyme.

Unlike other metalloproteinases, the human MMP-13 gene can be transcribed into proteins with different activities and functions. A proteolytic cascade that includes MMP-14 and

MMP-2 is activated by MMP-13. Different agents may regulate the MMP-13 transcription, especially growth factors, proinflammatory cytokines, and mechanical stimuli. Studies have shown that MMP-13 is a metalloproteinase much more complex than originally thought. Although our understanding of the biochemistry and regulation of MMP-13 has advanced greatly over the years, much remains unknown (Tardif et al., 2004).

Although MMP-13 (collagenase 3) was first discovered in breast cancer (Freije et al., 1994), it remains a metalloproteinase with a major involvement in inflammatory diseases such as rheumatoid arthritis and osteoarthritis, where it is associated with the resorption and destruction of bones and cartilage (Goldring et al., 2011). This metalloproteinase is also expressed by various periodontal cells and inflammatory cells in association with chronic periodontal disease (Hernandez et al., 2006).

MMP-13 and its relationship with periodontal disease. The main cells where MMP13 was detected were: fibroblasts, osteoblasts, macrophages, plasma cells, and gingival epithelial cells. MMP-13 has also been found to be involved in periodontal tissue destruction and alveolar bone resorption, along with MMP-9. The mechanisms of MMP regulation may be different, depending on the specific tissue and microenvironment. During the development of periodontal disease, these MMPs can be activated independently or together with pathogens and host proteases. (Leppilahti et al., 2014).

Previous studies have shown increased concentrations of MMP-13 in saliva, especially in females, which have been associated with an increased attachment loss. The detection of MMP-13 in gingival fluid showed increased levels of this proteinase in patients with chronic periodontitis compared to healthy subjects. In saliva, however, the MMP-13 amount was high in localized periodontitis but low in generalized periodontitis (Gursoy et al., 2013). MMP-13 has been associated with the destruction and resorption of bone and cartilage in periodontitis, being responsible for the activity of osteoclasts.

Several mechanisms have been reported, including osteoclast-secreted proMMP-9 activation, which will further digest the denatured collagen derived from the MMP-13 activity; cleavage of galectin-3, a known inhibitor of the osteoclastogenesis expressed on the surface of osteoclasts, which results in the abrogation of its inhibitory effect; and, last but not least, by adjusting the RANKL/osteoprotegerin (OPG) axis, thus favoring RANKL and TGF-β1 signaling. The conclusion of previous research was that MMP-13 is involved in osteoclast differentiation but also in breast cancer and bone metastases. (Pivetta et al., 2011).

MMP promoter activities can be stimulated by various proinflammatory cytokines, growth factors, the extracellular matrix metalloproteinase inducer (EMMPRIN), and bacterial virulence factors. Periodontitis-specific cells, i.e., gingival fibroblasts, epithelial cells/keratinocytes, osteoblasts/osteoclasts, periodontal ligament cells, together with recruited inflammatory cells, i.e., the neutrophils, monocytes/macrophages, and plasma cells, can thus be stimulated or diminished by various cytokines. For example, transforming growth factor (TGF-β) can suppress the transcription of MMP-1, -3, and -8 genes, but it can also induce MMP-13 gene expression. Pathological growth and activation of MMPs can trigger a chain cascade of proinflammatory factors in the gingival tissue affected by periodontitis and in the gingival

crevicular fluid. Furthermore, MMP-13 (collagenase-3) expression in gingival tissue sections was significantly increased in patients with chronic periodontitis, suggesting that MMP-13 expression is important in the proliferation of periodontal damage, in the progression of attachment loss, and in the deepening of periodontal pockets (Sorsa et al., 2006). MMP-13 is capable of inducing proMMP-9 activation and MMP-13 self-activation by in vitro auto-proteolysis. In vitro studies have also shown that MMP 9 can be activated by proMMP-2 and proMMP-13 (Rios et al., 2009). MMP-14, on the other hand, could activate MMP-8 and -13 in periodontitis sites. Thus, all these mechanisms could lead to an increase in the degradation of periodontal tissue and, consequently, to the progression of chronic periodontitis.

In addition, nonproteolytic oxidative activation of MMPs appears to play a central role in periodontal inflammation. At the level of periodontal tissues, reactive oxygen species (ROS) are able to activate key MMPs by oxidation of the enzymes using both direct and indirect mechanisms. Periodontopathogenic bacteria, cytokines, and prostaglandins lead to the degranulation of neutrophils, resulting in the release of myeloperoxidase (MPO). In addition to its antimicrobial activity, MPO is involved in regulating catabolism and connective tissue degradation by altering the protease/anti-protease balance.

Previous research has shown another possible mechanism involved in the development and progression of periodontal disease, namely, the activation of MMP mediated by reactive oxygen species (ROS). Oxidative stress increases the turnover of the extracellular matrix mediated by MMP-2, -9, and -13 in fibroblast and tumor cells (Siwik, Pagano and Colucci, 2001). Although there is considerable research on the importance of MMP-13 in the progression of periodontal disease, the exact mechanisms of association of periodontitis with this metalloproteinase are still unclear.

- **MMPs Inhibitors and Biomarkers in Periodontal Disease**

Among other molecules, the MMP inhibitors adopted in periodontal therapy are modified tetracyclines. Tetracyclines are antibiotics capable of inhibiting connective tissue degradation. Inhibitors are obtained by the chemical modification of molecules in the tetracycline family after the separation of antibiotic and protease inhibitor activities (Sczepanik et al., 2020). Indeed, serum levels of MMPs can be lowered with the administration of sub-antimicrobial doses of antibiotics such as doxycycline or tetracycline. For example, tetracycline (CT) can reduce MMP-8 and -9 activity in GCF and gingival tissue, even at a much lower dose than a traditional antimicrobial dose used in conventional therapy (Ramseier et al., 2009; Lahdentausta et al., 2018).

Minocycline provided the first evidence of inhibiting MMP by CT and its derivatives in the oral environment since it inhibits the collagenolytic activity of gingival crevicular fluid in the absence of bacteria.

The CTs have cationic chelating properties that inhibit MMP activity in the extracellular environment and prevent the activation of proMMP through oxidation by eliminating the reactive oxygen species (Yang et al., 2019). The TC also decreases transcriptional levels of MMP at an intracellular level.

Doxycycline hyclate is a low-dose tetracycline analog lacking antimicrobial activity, and it is indicated for the treatment of periodontal disease, acting by inhibiting the mechanisms of the MMP-8 and MMP-13 protease. The therapeutic effect of this antibiotic is due to the modulation of the host response since low-dose formulas do not exert antimicrobial activity (Steinsvoll, 2004). Tetracyclines have been found to inhibit MMP activity through cationic binding proteins, and their use in combination with mechanical periodontal therapy is widely accepted. In adults with periodontitis, low-dose doxycycline is currently used as adjunctive therapy in order to inhibit MMP activity because it significantly reduces the severity of periodontal disease, including alveolar bone loss.

Chemically modified tetracyclines (CMTs) do not have the 4-dimethylamino group of TC, which exerts antibacterial activity. Several CMTs with a range of potency and specificity for MMP's inhibition have been developed (Sadowski and Steinmeyer, 2001). For example, CMT-3 and -8 are the most potent inhibitors of MMP, with collagenase and CMT 3 being the only CMT with demonstrated efficiency against MMP- 1 (Uitto et al., 1994). It has also been found that various MMPs are inhibited by chlorhexidine (CHX). CHX is a biguanide chemical substance that provides effective antiseptic effects and is used to control plaque and reduce gingival inflammation. Several studies have shown that chlorhexidine directly inhibits MMP-2, MMP-8, and MMP-9, probably through a chelating mechanism.

CHX has been shown to dose-inhibit the collagenolytic activity of MMP-8 released by the human polymorphonuclear leukocytes triggered by forbol-12-myristate-13- acetate (PMA) (Collins et al., 2018). Taken together, these findings show that analysis of salivary levels of various risk mediators, such as metalloproteinases, are promising approaches used for the detection of early stages of various pathologies, both oral and systemic. There are numerous biomarkers involved in the complex pathological mechanism of periodontal disease (Figure I.2.3).

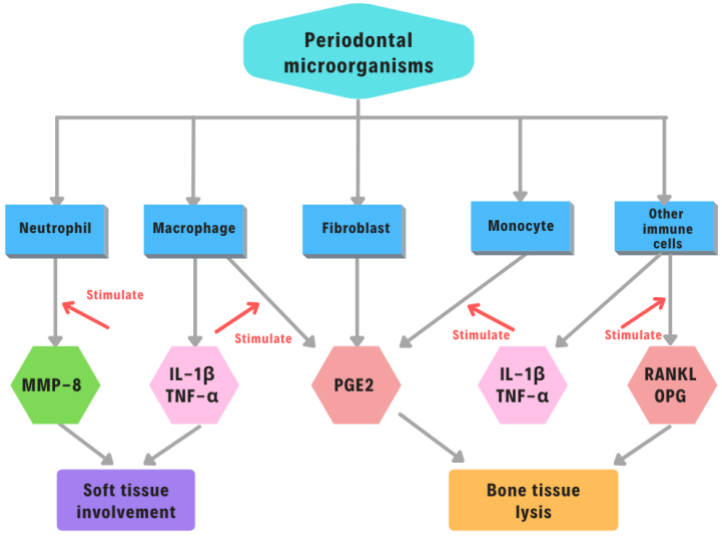


Figure I.2.3. Biomarkers in periodontal disease. Abbreviations: IL-1_β, interleukin -1; TNF-α, tumor necrosis factor α; PGE2, prostaglandin E2; RANKL, receptor activator of nuclear factor kappa-B ligand; OPG, osteoprotegerin.

For example, TNF-α is part of the major inflammatory cytokines, which are typically produced at the inflammation site by infiltration of mononuclear cells. TNF-α is a pleiotropic cytokine that can improve the host's defense mechanism by mediating inflammation and increasing cellular immune function (Schwartz, 2002; Boyce et al., 2005). At the same time, it can induce various pathological conditions due to TNF-α toxicity, causing tissue damage (septic shock syndrome, cachexia, autoimmune diseases, rheumatoid arthritis. meningococcal sepsis) (Schwartz, 2002; Boyce et al., 2005).

Salivary TNF-α levels can represent a valuable and accurate marker, both for aggressive and chronic periodontitis. Salivary PGE2 and IL1β can be considered essential mediators that play a key role in the pathogenic process of periodontal disease. They are strong stimulators of bone resorption and are produced by the cells of the human periodontal ligament in response to mechanical stress. PGE2 not only mediates inflammatory responses such as the increase of vascular permeability and dilation but can also work as strong stimulators of bone resorption and formation. This dynamic mechanism can be influenced by the concentration of PGE2 (Luchian et al., 2016). On the other hand, the RANK/RANKL/OPG system plays a significant role in the activation of osteoclasts, which are well known for their capacity to upregulate bone resorption (Teodorescu et al., 2019). However, periodontitis cannot be distinguished between mild, moderate, and severe forms based exclusively on RANK/RANKL/OPG, but it may be used as a complementary diagnostic tool for early diagnosis (Teodorescu et al., 2019).

- **MMPs and Peri-Implantitis**

Dental implants are increasingly used for partial or complete edentulism. Periimplantitis and peri-mucositis, two pathological conditions that affect tissues surrounding dental implants, are highly prevalent, representing approximately 25% and 43%, respectively, of patients with

implants (Derks and Tomasi, 2015). They are characterized by inflammation of the peri-implant connective tissue, leading to the progressive loss of supporting alveolar bone, which can cause more destructive lesions than periodontitis (Schwartz, 2002).

As such, peri-implant sites show large inflammatory processes with increased M1 type tissue destructive macrophages and neutrophils and high levels of leukocyte-type collagenases such as MMP-8 in the peri-implant sulcular fluid (PISF). This led to the development of point-of-care tests using MMP-8 as a reference test for grading peri-implantitis. Low MMP-8 levels were associated with periodontal and peri-implantitis health while the upregulation of MMP-8 levels denoted an increased risk for inflammation (Araujo and Lindhe, 2018). The MMP-8 tests proved to be more sensitive and reliable than other biomarkers such as calprotectin, gelatinases, neutrophil elastase, myeloperoxidases and MMP-9, although PISF MMP-9 also is a good marker for tissue health surrounding the implant (Golub et al., 2020).

Both MMP-8 and MMP-9 levels were associated with the degree of healing process and osseointegration, indicating their functional role in tissues around the implant. For example, higher levels were detected during or immediately after implantation compared to post-implantation and healing (Kaliyatz et al., 2020). Although MMP-8 is considered as the main collagenase in active peri-implantitis (Arakawa et al., 2012), MMP-9 has also been shown to be involved in the pathogenesis of peri-implantitis (Zhang et al., 2020) via a LOX-1 (lectin-like oxidized low-density lipoprotein) and Erk1/2 (extracellular signal-regulated protein kinase) mechanism that has been suggested as a potential target for decreased inflammation and increase apoptosis in peri-implantitis. In addition, MMP-1 may be involved in the pathogenesis of peri-implantitis, given its increase in fibroblasts from peri-implantitis (Irshad et al., 2013) and reduced gene expression of tissue inhibitors of matrix metalloproteinases (TIMP-1) (Verstappen and Von den Hoff, 2006), which may indicate a loss of attachment around dental implants (Ramseier et al., 2016). It is interesting that a polymorphism in the MMP-1 promoter (G-1607GG) involved in transcription has been reported in implant failure (Leite et al., 2008).

A similar polymorphism for the promoter region of the MMP-8 gene (C-7997) (Costa et al., 2013) and MMP-13 (-77 A < G) has been associated with loss of osseointegration and implant loss (Ferrer et al., 2021).

Periodontitis represents one of the most prevalent chronic inflammatory oral diseases. It is characterized by an inflammatory destruction of periodontal attachment complex leading to irreversible loss of bone and tooth-supporting tissue (Kononen et al., 2019; Kim and Kim 2021). Among the components of periodontium lost is type I collagen, found primarily in periodontal ligament and alveolar organic matrix (Benjamin 2010; Papapanou and Susin 2017).

The destruction of periodontium is mediated by the plasminogen-dependent, phagocytic, osteoclastic, and matrix metalloproteinase (MMP) pathways (Uitto et al., 2003; Minervini et al., 2020). The MMPs, a family of zinc and calcium-dependent proteolytic enzymes, which are secreted by immune cells in response to inflammatory stimuli, are considered the most important in mediating the degradation of the extracellular matrix (Boelen et al., 2019; Moccia et al., 2020) and have been recognized as important biomarkers in the early detection of several diseases (Laronha and Caldeira 2020).

Normally, these enzymes are tightly regulated and play a critical role in bone morphogenesis and tissue repair (Birkedal-Hansen 1993; Sorsa et al., 2004). However, in pathological conditions such as periodontitis, these enzymes are involved in the destruction of extracellular matrix components such as collagen, elastin, fibronectin, laminin, and entactin (Narayanan and Page 1983; Rathnayake et al., 2015).

This results in oral pathological processes such as the destruction of the periodontal tissue, tumor invasion, and dysfunctions of the temporomandibular joint (TMJ) (Aiba et al., 1996; Escalona et al., 2016). Among these several groups of proteinases, the zinc-metalloproteinases such as matrix metallopeptidases 9 (MMP-9) contain a Zn ion in the catalytic domain. Moreover, MMP-2 and MMP-9 may have a binding domain for gelatin, inserted between the catalytic and the active domain, and this is the reason of why MMP-9 is also called gelatinase B (Laronha and Caldeira 2020). MMP-9 or gelatinase B is primarily found in saliva and gingival crevicular fluid; it is present in dental tissues with numerous active forms, weighing 82–132 kD, and is involved in inflammation, wound healing, and tumor growth (Sorsa et al., 2006).

The involvement of MMP-9 both in periodontal disease and in the orthodontic periodontal reshaping has already been demonstrated by several studies (Marcaccini et al., 2010; Grassia et al., 2014; Grant et al., 2013; Lahdentausta et al., 2018). Both in vivo and in vitro evidence show that orthodontic dental movement causes mechanical stress (Maspero et al., 2019a; Maspero et al., 2019b), which, in turn, generates biochemical and structural responses in a diversity of cell types (Perinetti et al., 2015; Alikhani et al., 2018; Lahdentausta et al., 2018).

As such, the early stage of orthodontic dental movements involves an acute inflammatory response featuring local tissular ischemia, periodontal vasodilatation, and the migration of leucocytes through the capillaries of the periodontal ligament (Di Domenico et al., 2012). Elevated MMPs have been associated with increased inflammation and loss of tooth-supporting tissue present in periodontal disease, while periodontal treatment decreases inflammation and lowers MMP-9 levels (Goncalves et al., 2013; Meschiari et al., 2013; Balli et al., 2014; Alikhani et al., 2018).

Orthodontic tooth movement (OTM) involves comprehensive periodontal and alveolar bone remodelling (Bildt et al., 2009; Meeran, 2013). It is considerably different from physiological tooth movement (Isola et al., 2016) since it begins with an inflammatory-like response that involves the activation of different biological factors and degradation/synthesis of the extracellular matrix (ECM) in the periodontal ligament (PDL) (Xu et al., 2020). The key trigger factor responsible for OTM is the pressure exerted on PDL cells and the extracellular matrix, which causes changes in the gene expression within cells and the extracellular matrix, and also induces the release of specific cytokines and chemokines. In response to mechanical loading, cytokines and chemokines control alveolar bone remodelling.

Orthodontic forces induce capillary vasodilatation in the periodontal ligament, resulting in inflammatory cell migration and cytokine production (Meeran, 2013). Matrix metallo-proteinases (MMPs) are a family of proteases that are important in remodelling the extracellular matrix

(ECM) (Bildt et al., 2009). A total of 23 human MMPs have been reported to date (Snoek-van Beurden and Von den Hoff, 2005). Among these, metalloproteinase-8 (MMP-8) and metalloproteinase-9 (MMP-9) are members of the collagenase and gelatinase groups, respectively (Snoek-van Beurden and Von den Hoff, 2005). They are initially synthesized as inactive proenzymes that may be stimulated in the ECM by proteolytic processing (Snoek-van Beurden and Von den Hoff 2005).

MMPs and TIMPs together play a major role in periodontium physiological remodelling (Ejeil et al., 2003) and the response to mechanical forces during orthodontic treatment (Ingman et al., 2005). MMP inhibition by synthetic MMP inhibitors has been shown to decrease OTM (Holliday et al., 2003). MMP-8 is deposited in an inactive form, specifically in granules of polymorphonuclear leukocytes (PMNs), and is mainly thought to be regulated by its selective granular release from triggered PMNs at inflammation sites (Apajalahti et al., 2003).

Additionally, MMP-8 (human neutrophil collagenase, collagenase-2) is also released by certain non-PMN lineage cells, such as gingival fibroblasts, bone, and plasma cells (Hanemaaijer et al., 1997). MMP-8 is the most effective in hydrolyzing type I collagen (Hasty et al., 1987) and is the primary interstitial collagenase in inflamed human gingiva (Ingman et al., 2005).

A healthy periodontium is crucial to prevent any unsatisfactory changes to the tissues that support the teeth (Haas et al., 2014). Pathogenic bacteria in close contact with the gingival margins are the key etiological agents for the development of periodontal disease (Botero et al., 2015). Gingivitis is a periodontal disease that manifests without periodontal attachment loss; however, it exhibits a change in the equilibrium between the biofilm and the host. Gingivitis can progress to periodontitis, which is associated with attachment loss and bone loss (Botero et al., 2015). Fixed orthodontic devices may increase supragingival biofilm accumulation and degrade periodontal health (Haas et al., 2014), increasing the amounts of pathogenic anaerobic bacteria in supra or subgingival biofilms during orthodontic therapy. Therefore, proper hygiene is needed to prevent the development of gingivitis and periodontitis.

Although several biomarkers have been considered for the diagnosis of periodontal disease, there is no consensus regarding the biomarkers for monitoring bone resorption in orthodontic treatment. Bleeding on probing (BOP) can be readily evaluated and is useful for early diagnosis (Ainamo and Bay 1975) and prevention of periodontal disease since it precedes other clinically detectable signs of gingivitis (Muhlemann and Son 1971). Furthermore, it correlates with the severity of inflammatory conditions in the gingival tissue (Greenstein, Caton and Polson, 1981).

If persistently present during the monitoring period, it represents a significant prognostic factor for periodontal impairment at the level of a particular situs. BOP sites exhibit a greater probability of severe attachment loss when compared to non-bleeding sites (Schatzle et al., 2003). Substantial plaque accumulation and increased BOP are associated with orthodontic therapy (Al-Anezi 2015). Patients with high BOP are “at-risk” and demand a more rigorous periodontal therapy regimen than those with little to no BOP (Sebbar et al., 2015).

Since a majority of orthodontic patients will exhibit inflamed, swollen, bleeding gingiva at one point at least during treatment, suitable caution is required, and supportive periodontal care

should be routinely recommended as an essential component of orthodontic therapy (Sebbar et al., 2015). Reports have illustrated the value of a full-mouth examination at six sites per tooth for a detailed analysis of orthodontic patients' periodontal status (Jin 2007).

However, this approach commands a long and time-consuming clinical diagnosis that depends on the clinician's expertise. Moreover, this process must be repeated at regular intervals to determine the patient's periodontal status at recall visits. To the best of our knowledge, this is the first study to evaluate salivary biomarkers in patients undergoing orthodontic treatment before and after periodontal therapy.

This study aimed to evaluate the changes in the levels of matrix metalloproteinase-8 and matrix metalloproteinase-9 before and during orthodontic treatment and also after periodontal treatment and to analyze their correlation with the bleeding on probing index (BOP). Furthermore, we aimed to identify 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. To determine whether MMP-9 levels can reliably predict the chronic inflammatory oral diseases, in this study, we examine the effects of periodontal treatment on salivary MMP-9 levels in patients with stabilized pre-existing periodontal history, where treatment is done either without or coupled with orthodontic treatment.

1.6.2. Materials and Methods

⇒ First study

All the steps of this study were thoroughly explained to the patients prior to enrollment. The patients were instructed about the purpose of the study and provided informed consent before participating in the study. The study was conducted in accordance with the Helsinki Declaration. The protocol was approved by the Ethics Committee of the University of Medicine and Pharmacy from Iasi, Romania (Protocol identification code 29.01.2020/2540).

Subjects. This study 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. 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). The BOP score was evaluated as the proportion of bleeding sites (dichotomous yes/no evaluation) at six sites (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) at the bottom of the sulcus/pocket on all present teeth when stimulated by a standardized manual periodontal probe (Trombelli et al., 2018).

A single examiner, blinded to the purpose of the study, performed all measurements during the clinical evaluation. For the intra-examiner calibration, 20 non-study individuals with orthodontic treatment were selected. The intra-examiner reproducibility was 90%. A periodontal specialist, different from the examiner performed the periodontal treatment during the study.

Saliva Sampling. 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 jeune" 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 ºC 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 ºC to remove cellular and insoluble debris. The supernatant was then transferred in a new Eppendorf 1.5 mL tube and appropriately labeled. Following the pre-processing steps, all samples were kept at - 80 ºC until analysis.

The saliva samples were processed according to the manufacturer's instructions. 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. 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.

The Kolmogorov-Smirnov test was used to test the normality of data (sample size > 50 respondents), which is a prerequisite for many statistical tests because normal data is an underlying assumption in parametric testing. The normality of the data for MMP-8 and MMP-9 levels and BOP values was tested separately for each of the three phases: T1 (before application of the orthodontic treatment), T2 (after application of orthodontic treatment), and T3 (after application of orthodontic and periodontal treatment). The null hypothesis for this test is that the data are normally distributed, and it was accepted (p-value > 0.05) for MMP-8 (T3), BOP% (T1), BOP% (T2), BOP% (T3). For double confirmation, a normal Q-Q plot was used to graphically visualize the normal distribution of the variables. The Wilcoxon signed-rank test for nonparametric statistics was used to compare MMP- 8 (T3) with MMP-8 (T2), MMP-9 (T3) with MMP-9 (T2), MMP-8 (T3) with MMP-8 (T1), MMP-9 (T3) with MMP-9 (T1), and to determine for each of these measurements (MMP-8 and MMP-9) if the values at T3 were significantly lower than those at T2 and significantly higher than those at T1.

The paired t-test (parametric test), the equivalent of the Wilcoxon Signed Test for parametric variables, was used to compare the BOP (T3) with BOP (T2) and the BOP (T3) with BOP (T1), and to determine if the values at T3 were significantly lower than those at T2 and significantly higher than those at T1. Pearson and Spearman correlations were used to test whether there was a statistically significant linear relationship between MMP-8 levels and BOP values for all three stages: T1, T2, and T3.

A significantly strong relationship was found between the two measurements at T2 (Spearman's rho = 0.939, p-value < 0.001) and T3 (r = 0.842, p-value < 0.001) and a medium but statistically significant correlation was observed at T1 (Spearman's rho = 0.614, p-value < 0.001). For a graphical visualization of this relationship, we used a scatter plot. Spearman's correlation was used to test whether there was a statistically significant linear relationship between MMP-8 and MMP-9 levels at all three stages: T1, T2, and T3. A significant and medium relationship was found between the two measurements at T3 (Spearman's rho = 0.440, p-value < 0.01) and at phase T2 (Spearman's rho = 0.239, p-value < 0.05). For a graphical visualization of this relationship, we used a scatter plot. The same type of correlation was used to test whether there is a statistically significant linear relationship between MMP-9 and BOP for all of the three stages: T1, T2, and T3. Two significant and medium relationship were found at phase T2 (Spearman's rho = 0.314, $p < 0.01$) and T3 (Spearman's rho = 0.426, $p < 0.01$).

Descriptive analyses were used to calculate descriptive coefficients such as mean, standard deviation, minimum, and maximum for all the variables included in the sample. The box plot was used to graphically visualize the difference between means and distribution for each of the three measurements (MMP-8, MMP-9, and BOP) within the three stages: T1,T2, and T3. In order to determine the ROC (receiver operating characteristic) curve, we divided the patients into three groups: healthy group (BOP < 10%), localized gingivitis group (BOP $\geq$ 10% and BOP $\leq$ 30%), and generalized gingivitis group (BOP > 30%) (Trombelli et al., 2018) and calculated the cut-off point for MMP-8 and MMP-9 in all three stages: T1, T2, and T3.

$\Rightarrow$ Second study

Subjects. The study was performed under the Institutional Review Board protocol no. 5329/2018 approved by the ethics committee of the Grigore T. Popa University of Medicine and Pharmacy, and signed informed consent was obtained from each participant in the study. The sample population included in the current research consists of consecutive patients that were selected in a 12-month interval. Sixty individuals of which 32 males and 28 females in good general health from 21 to 38-year-old were enrolled in the study. The following inclusion criteria were used: a minimum of 20 teeth in functional dentition, moderate or severe periodontitis and without periodontal treatment at the time of enrolment.

Periodontal exam included the recording of periodontal pocket depths (PPD) from six sites of each tooth. The six probing sites were distributed as follows: three sites on the buccal surfaces (mesial, central, and distal) and three sites on the lingual surfaces of the teeth (mesial, central, and distal). Bleeding on probing was recorded and the sulcus bleeding index (SBI) was determined.

A complete periodontal probing was performed using an electronic probe (PaOn®, Orange Dental, Biberach a. d. Riss, Baden-Würtemberg State, Germany), and data were transferred using additional software. The following exclusion criteria were applied: smoking, heavy drinkers, immunocompromised, use of anti-inflammatory drugs or other medication affecting the periodontium, use of antibiotics and steroids, and a history of systemic and infectious diseases.

The sixty patients included in the study were randomly divided in three groups as follows: group 1, a control group that included 16 (7 men, 9 women) subjects without periodontal disease and/or clinical gingival modifications; group 2, which included 22 subjects (10 men, 12 women) with periodontal disease (chronic periodontitis localized in minimum 3 teeth) who received periodontal treatment (PD); and group 3, which included 22 (11 men, 11 women) subjects with periodontal disease (chronic periodontitis localized in minimum 3 teeth), who received both periodontal and orthodontic treatment (POD).

All individu als received periodontal examination and were diagnosed based on the clinical criteria established by the American Academy of Periodontology (Greenwall, 2001). All patients underwent treatment including oral hygiene instructions. Periodontal treatment was identical for both PD and POD groups and consisted of supra- and subgingival scaling and root planning over a maximum 4-week period.

The subgingival scaling was performed using the same ultrasonic device (Acteon Satelec®, Mérignac, Gironde, France), and the same type of subgingival inserts while for root planning area specific curettes (Hu-Friedy®, Chicago, IL, USA) were used. Therapeutic treatments were performed every 8 weeks starting 10 days after study commencement. For orthodontic treatment, we used metallic fixed appliances that were bonded after the periodontal status stabilized. No dropouts occurred prior or during the treatment.

Saliva Sample Collection and Analysis. Saliva was collected at two time points from patients included in PD and POD groups as follows: for the PD group, one initial baseline collection and a second collection 6 months following completion of periodontal treatment; for the POD group, one initial baseline collection and a second collection 6 months after completion of periodontal treatment and stabilization of the orthodontic treatment. The timing for the second saliva collection in the POD group was delayed compared to the PD group to allow for an evaluation of the effects of the orthodontic treatment on the inflammatory markers level once the treatment has stabilized. One baseline saliva collection was performed in the control group at the beginning of the study. All samples were collected by one investigator to ensure consistency in the protocol. Saliva was collected without stimulating its secretion from the salivary glands (i.e., paraffin gum or citric acid) in order to avoid any interference of the stimulating agent on marker release.

Collection was carried out using Eppendorf tubes placed on ice; particular attention was given to contamination with blood, since MPP is also present in blood and its levels were shown to be different (i.e., higher) in blood compared to those present in the salivary fluid. Samples suspected of blood contamination were discarded. Samples were stored at -20°C pending analysis. The levels of MMP-9 in collected saliva samples were measured via ELISA

immunoassay following the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN, USA) (Marcaccini et al., 2010).

Statistical Analyses. Statistical analyses were performed using SPSS 24.0 for Windows (IBM Corporation, North Castle Drive, Armonk, NY, USA). The Kruskal–Wallis test was applied to determine the differences in MMP concentrations between groups of patients according to treatment and clinical parameters. The Newman–Keuls post hoc test was also applied for the pair analysis of two groups of patients. MMP-9 values were reported as mean values and standard deviation.

The evolution of MMP-9 values was also presented in %, with the proportion being represented by the decrease of the MMP-9 value related to the value registered before the treatment. The univariate correlational analysis was performed based on the Spearman rank order correlations test. To better highlight the effect of the treatment and the degree of malocclusion, the graphs were generated using STATA 16.1 (StataCorp LLC., College Station, TX, USA). A p-value of less than 0.05 was considered statistically significant.

1.6.3. Results

⇒ First study

We analyzed 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.2.2.).

Table I.2.2.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)	0.450 ± (0.48) ng/mL
MMP9(T2)	1.899 ± (1.82) ng/mL
MMP9(T3)	0.100 ± (0.07) ng/mL *#
MMP8(T1)	0.100 ± (0.07) ng/mL
MMP8(T2)	0.267 ± (0.20) ng/mL
MMP8(T3)	0.140 ± (0.08) ng/mL *#
BOP(T1)	5.088 ± (2.72)%
BOP(T2)	16.224 ± (8.84)%
BOP(T3)	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.

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.2.4., Table I.2.2.).

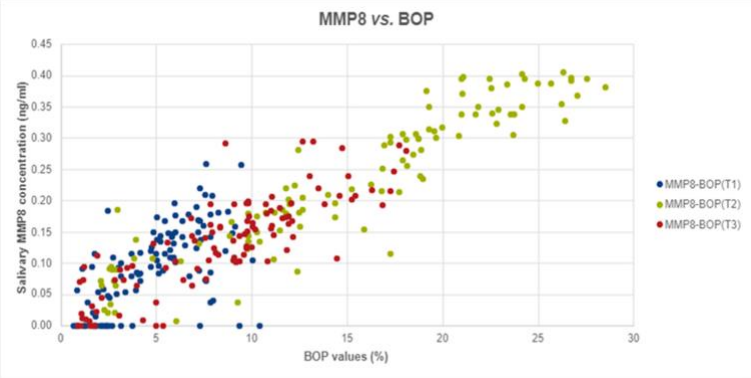


Figure I.2.4. The correlation between salivary MMP-8 and BOP values in T1, T2, and T3.

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.2.3.), also in Figure 2, 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.2.5.).

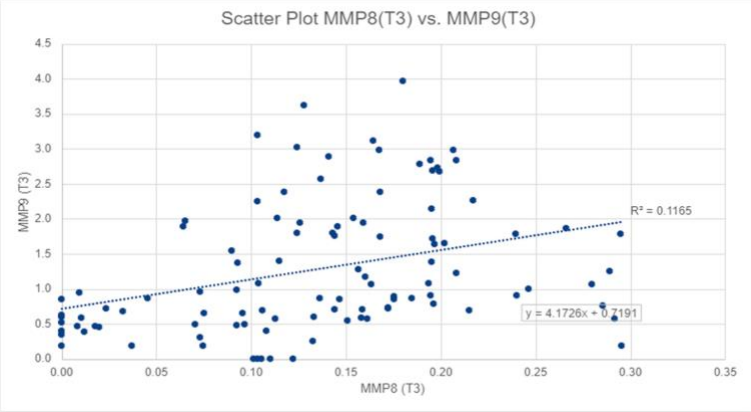


Figure I.2.5.The correlation between salivary MMP-8 and MMP-9 values in T3

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 I.2.3).

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 1). 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 1. 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 3.

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.2.3.).

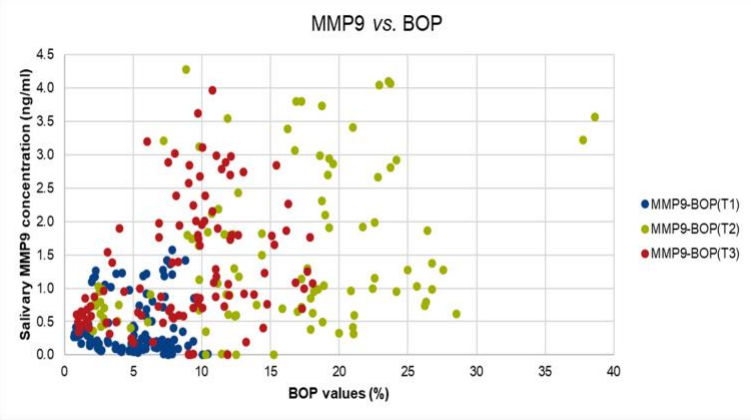


Figure I.2.6. The correlation between salivary MMP-9 and BOP values in T1, T2, and T3.

Table I.2.3. 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	0.249 ± 0.09 **	0.994 ± 0.26 ##**
Bleeding on probing (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.

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.2.4., Figure I.2.7.).

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.

We conducted ROC analysis in order to determine a cut-off for MMP-9, between healthy versus localized gingivitis group. The results reveal an optimal cut-off using the Youden index method of 0.874 ng/mL (for MMP-9) for which we have a sensitivity of 73.2% and a false positive of 30.3% (Table I.2.5., Figure I.2.8.).

Table I.2.4. Results from ROC analysis of individual salivary biomarker levels comparing healthy group to 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

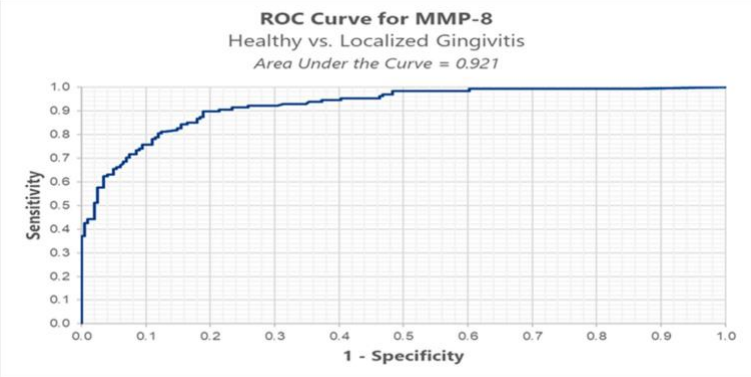


Figure I.2.8. ROC analysis of MMP-8 in healthy versus localized gingivitis.

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.2.9).

Table I.2.5. Results from ROC analysis of individual salivary biomarker levels of MMP-9 comparing the healthy group to the localized gingivitis group

Group	Optimal Cut-Off	Sensitivity	Specificity	FP	FN	AUC
MMP-9 (Healthy versus Localized Gingivitis)	0.874 ng/mL	0.732	0.697	0.303	0.268	0.752

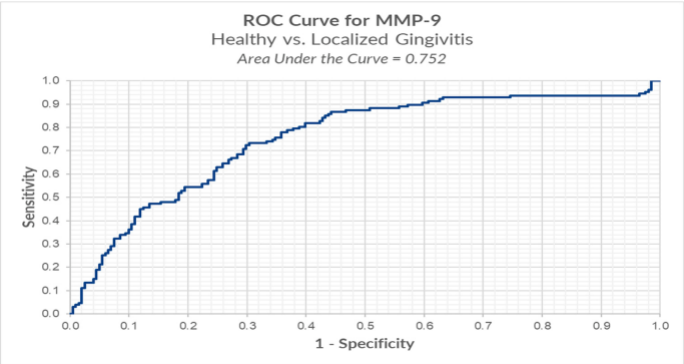


Figure I.2.9. ROC analysis of MMP-9 in healthy versus localized gingivitis.

⇒ Second study

Clinical Parameters. Analysis showed a significant difference before treatment between PD and control groups for PPD (4.18 ± 0.21 vs. 1.65 ± 0.14, $p < 0.0001$) and SBI (2.9 ± 0.11, vs. 0.31 ± 0.11, $p < 0.0001$) as well as between POD and control groups for the same parameters: PPD = 4.54 ± 0.17, $p < 0.0001$; SBI = 3.45 ± 0.12, $p < 0.001$. Periodontitis treatment significantly improved PPD (3.23 ± 0.19, $p < 0.01$) and SBI (2.04 ± 0.12, $p < 0.01$) while periodontitis combined with orthodontic treatment had a greater effect compared to the control group and the PD group's treatment for both clinical parameters, i.e., PPD = 2.4 ± 0.1, SBI = 2.04 ± 0.12, $p < 0.0001$ for both.

3.2. Effects of Periodontal and Combined Periodontal with Orthodontic Treatment onMMP-9 Levels Patients with untreated periodontal disease had significantly higher levels of salivary MMP-9 compared to controls (control group: 155.6 ± 38.63 ng/mL; PD: 582.27 ± 48.2 ng/mL; POD group: 602.55 ± 64.55 ng/mL; $p < 0.0001$ for both).

However, following intervention, periodontal treatment alone lowered MMP-9 significantly compared to the levels before treatment (17.3% reduction; $p = 0.0046$). A combination of periodontal with orthodontic treatment drastically decreased MMP-9 levels by 42.3% compared to pretreatment levels ($p < 0.0001$). Although MMP-9 levels dropped significantly after treatment in both PD and POD groups, there was a significant difference ($p = 0.00012$) in MMP-9 levels following each of the two treatments, with the treatment for the POD group having the most significant effect in lowering MMP-9 levels compared to the PD and control groups ($p = 0.0005$) (Figure I.2.10.).

The Effect of Malocclusion on MMP-9 Levels. The degree of malocclusion significantly affected salivary MMP-9 levels. Because of this, prior to treatment, patients with periodontal disease had MMP-9 values that differed significantly depending on the angle class (PD group: $p = 0.005$; POD group: $p = 0.003$); this effect was more pronounced in patients with angle class II/1 and II/2 (Figure I.2.11).

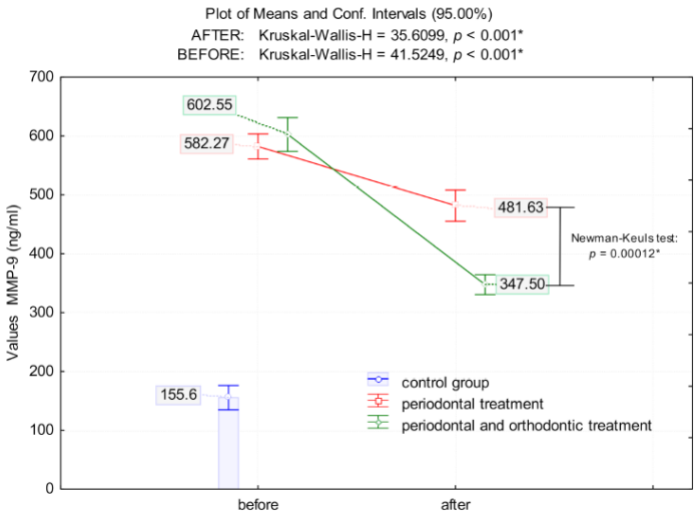


Figure I.2.10. The means value of MMP-9 [ng/mL] in patients with periodontal disease prior and after periodontal treatment (PD) and periodontal and orthodontic treatment (POD) treatment; (*) indicates that marked effects are significant at $p < 0.05$.

Following the PD group's treatment, MMP-9 decreased significantly compared as a function of the angle class ($p = 0.03$). The treatment in the POD group significantly lowered MMP-9 levels compared to those of the PD and control groups ($p < 0.0001$); however, there were no significant differences between the angle classes ($p = 0.176$) (Figure I.2.11.).

Correlation Between Clinical Parameters and MMP-9 Levels. Before treatment, the Spearman rank analyses showed a significant positive association between the probing pocket depth (PPD) and MMP-9 levels in POD patients ($r = 0.47$, $p = 0.03$) with no difference in the PD group ($r = 0.033$, $p = 0.88$).

Following intervention, MMP-9 levels were significantly correlated with PPD in the PD group ($r = 0.33$, $p = 0.029$) but not in the POD group ($r = 0.678$, $p = 0.764$). Similarly, there was a significant correlation between SBI and MMP-9 levels before treatment in the POD group ($r = 0.47$, $p = 0.032$) but not the PD group ($r = 0.08$, $p = 0.71$). After treatment in the PD group, MMP-9 levels in the PD group were significantly correlated with SBI ($r = 0.46$, $p = 0.034$) while the treatment in POD group abrogated the correlation between SBI and MMP-9.

1.6.4. Discussion

The findings of first 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 (Ingman et al., 2005). This could explain the strong positive correlation found in our study between MMP-8 levels and BOP values.

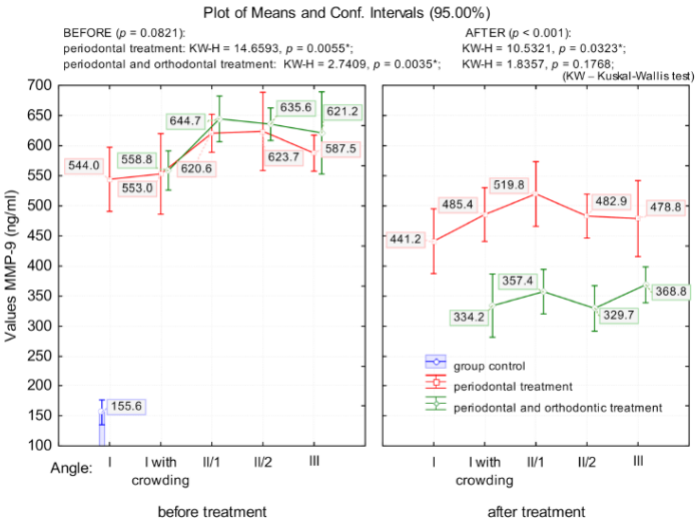


Figure I.2.11.The means value of MMP-9 in patients with periodontal disease, in relation to angle class; (*) indicates that marked effects are significant at $p < 0.05$.

Periodontal complications are one of the most frequent adverse effects of orthodontic treatment (Dannan 2010), 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 various studies (Mattingly et al., 1983; Perinetti et al., 2004).

Trombelli et al. (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 (Lang et al., 1990; Chapple, 1997), 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 (Daly et al., 2001), 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 (Galgut 1999).

As other studies have shown, BOP is used to evaluate the results of preventive and treatment strategies for periodontal diseases (Lang et al., 1986). Nonetheless, oral fluid biomarkers exhibit the ability to provide further, more accurate insight when compared to regular clinical investigations (Giannobile et al., 2009). Moreover, those investigations (BOP, plaque index, probing depth, clinical attachment level, and radiographic recordings (Buduneli and Kinane 2011) illustrate only retrospective data, and not the current disease status (Armitage,

2004; Buduneli and 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., 6AD).

Various studies have shown that increased MMP-8 and MMP-9 levels characterize not only periodontal disease (Sorsa et al., 2006; Sorsa et al., 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 (Rai et al., 2008; Boşca 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 respectively0.874 ng/mL. This is the first study to analyze 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 (Sexton et al., 2011; Boşca 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 (Mantyla et al., 2006; Sexton et al., 2011; Boşca 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., 2006). MMP-8 is the key collagenolytic component found in the gingival tissue and oral fluids (Hernández-Ríos 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. (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. (Mantyla et al., 2003). Interestingly, Marcaccini et al. (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 analyzed as a biomarker before and during orthodontic treatment in order to identify the individual periodontal inflammatory status and disease prognosis.

The results of our second study show that both periodontal and the combination of periodontal and orthodontic treatments were effective in significantly lowering MMP-9 levels compared to patients who did not receive the treatment. However, it was the combination of periodontal with orthodontic treatment that had the greatest effect on MMP-9 levels compared to periodontal or control groups alone.

Furthermore, while the treatment in both the PD and POD groups significantly improved clinical parameters, the POD group treatment had the greatest effect. This improvement of clinical parameters following intervention in the PD and POD groups was positively associated with MMP-9 levels. Finally, the degree of malocclusion significantly affected the effect of the treatment on MPP-9 levels with PD treatment having the most pronounced effect.

Periodontitis is one of the most prevalent inflammatory pathology affecting nearly half of people over 30 years old (Papapanou and Susin 2017). It is characterized by an immune inflammatory process ultimately leading to the destruction of periodontal attachment and supporting tissue and bone resorption. Several periodontal bacteria together with microbial proteases such as metalloproteases including host-derived MMPs, all participate in the process leading to progression of periodontitis, tissue, and ligament degradation.

Among them are *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*; together with the host's genetics and other environmental factors, these bacteria are active contributors in the infections and inflammatory processes that result in the production of metalloproteases, including host MMPs (Sorsa et al., 2006; Cobzeanu et al., 2017; Rathnayake et al., 2017). MMP-9 is among the best studied proteinases when it comes to its role in periodontitis and its activation in infections.

In the second study, salivary MMP-9 levels were associated significantly with periodontal and orthodontic treatment and differentiated from the control group. Therefore, MMP-9 can be used with high fidelity not only as a marker of periodontal inflammation but also as a tool for assessing the effectiveness of the nonsurgical periodontal and orthodontic therapy. The increased MMPs, whether in saliva or serum samples in periodontitis, were well documented. For example, salivary as well as circulating MMP-8 and MMP-9 levels were significantly elevated in periodontitis patients (Marcaccini et al., 2009) and reduced after periodontal treatment (Figueredo et al., 2004; Correa et al., 2008; Marcaccini et al., 2010).

The use of MMPs as salivary markers for local or systemic pathologies such as CVDs, diabetes, dyslipidemia has been of interest for sometimes, but not without challenges, given their vulnerability against local inhibitors in periodontitis condition that can limit their use in systemic diseases (Miller et al., 2010). In addition to the periodontitis group that was associated with increased MMP-9, patients who received both periodontal as well as orthodontic treatment displayed an augmentation in the inhibition of MMP-9 levels, compared with either treatment alone. To our knowledge, this is the first report demonstrating that combination of periodontal and orthodontic treatment is associated with a reduction in MMP-9 levels.

Previous work has shown increased salivary biomarkers in orthodontic treatment involving alignment with fixed appliances, which have been associated with tooth movement (Kapoor et al., 2014; Saloom et al., 2019). This is not surprising since there is increased osteoclast activity when orthodontic forces are applied, and MMP-9 is expressed in osteoclasts where it controls proteolysis in bone resorption. Therefore, inflammatory markers such as MMP-9 are associated with osteoclasts activity, a phenomenon present in response to orthodontic applied forces (Takahashi et al., 2006; Grant et al., 2013).

In vitro studies showed an increase in MMP-9 following application of orthodontic forces, an effect that is dependent on the degree of tension and compression (Kapoor et al., 2019). In our study, however, we measured MMP-9 levels after orthodontic treatment was completed and patients stabilized. This resulted in a further decrease in the MMP-9 levels in patients who received orthodontic treatment after periodontitis was improved or resolved.

These results are also in line with findings demonstrating that MMP-9 levels decreased significantly in patients with periodontitis with orthodontic restorations (Kushlinskii et al., 2012) and that MMP-9 levels oscillated during application of orthodontic forces and decreased as early as 24h after appliance activation (Capelli et al., 2011).

Another important aspect of the study is highlighted by the findings that the MMP-9 values following the combined treatment dropped significantly more for all angle classes, with no significant differences between groups, whereas in the case of periodontal treatment MMP-9 values remain elevated in the patients who had angle classes II and III malocclusions. A comparative analysis of MMP-9 values in relation to the type of treatment and to the angle class shows that after combined periodontal and orthodontic treatment, the values of MMP-9 lowered significantly more, despite the fact that they were significantly higher before treatment.

Therefore, although at the start of the treatment patients with periodontal problems who were about to begin periodontal treatment combined with orthodontic treatment had higher values

of MMP-9 (although not significant), these values dropped significantly more compared to those of patients who had only periodontal treatment. The persistence of high values of MMP-9 in patients who received only periodontal treatment, particularly in the case of those with angle classes II/2 and II/1 malocclusions, as well as those with angle class III malocclusions, is convincing and clearly demonstrates that orthodontic treatment combined with periodontal treatment significantly reduces inflammation in the affected periodontal tissues. A complex analysis to assess changes in MMP-9 values in chronic inflammatory oral diseases could be performed using both MMP-9 levels and the type of treatment, in a clustered form. This method could lead to a significant increase in the prediction of the evolution of periodontal disease (Boiculese, Dimitriu and Moscalu, 2009).

Existing literature shows that both the saliva tests and the tests performed on crevicular fluid provide valuable diagnosis information concerning the stage of the inflammation in periodontal disease. Several authors have shown that metalloproteinase-8 (MMP-8), an enzyme responsible for tissue destruction, was positively associated with periodontal disease (Kinane et al., 2003; Herr et al., 2007; Prescher et al., 2007). Because of this, an immunochromatography assay was developed and is commercially available for assessing MMP-8 in crevicular fluid. This test can be carried out in clinical practice and has the same accuracy as a laboratory test. This facilitates testing of this particular metalloproteinase and opens new perspectives in terms of the predictability of the chosen treatment plan (Sorsa et al., 2010). Currently, no similar test/assay exists for determining MMP-9 in saliva or in the crevicular fluid.

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.

1.6.5. Final remarks

In our studies 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.

Our results point to salivary MMP-9 as a strong candidate for quantifying inflammation in affected periodontal tissues during orthodontic treatment. It further indicates that MMP-9 can be used to accurately predict the level of inflammation associated with the type of malocclusion, which makes it a real and viable diagnosis instrument in monitoring the evolution of the periodontium during orthodontic treatment. Larger scale studies conducted on patients with various degrees of periodontitis and orthodontic treatments are needed to further establish the use of salivary MMP-9 as a predictor of inflammation following orthodontic treatment.

1.7. Associations between periodontal periodontal disease and specific pathogens by mRNA analysis.

1.7.1. Introduction

The pathogenic mechanism of periodontal disease is a complex interaction between plaque bacteria and a susceptible host, characterized by an inflammatory process that leads to the destruction of attachment tissues and bone loss. It is a widespread disease, with approximately half of people over 30 being affected, while the severe form affects approximately 11% of the global population and poses a high burden on healthcare systems, generating costs that reach billions of dollars each year. This is why there is a high need to find novel diagnostic assays and to better understand the pathogenic mechanisms (Preshaw et al., 2004). Thanks to data gathered from carefully conducted, longitudinal monitoring studies, the understanding of the prevalence of periodontal disease has changed.

The pathogenic mechanisms are now better understood, and this change has led to a shift from an older to a more recent theory. The old theory of periodontal disease was that periodontitis is an inevitable consequence of gingivitis, that it is uniformly distributed in the population, that disease severity is correlated with plaque levels, which lead to a linear, progressive loss of attachment over time, and that the severity of periodontitis increases with age.

By comparison, the new theory of periodontitis states that gingivitis and mild periodontitis are common (seen in about 40–60% of people), and approximately 10–15% of the population exhibit advanced periodontitis. Gingivitis precedes periodontitis, but not all sites with gingivitis develop periodontitis, and periodontitis is not a natural consequence of aging.

The most frequent periodontal pathogens recognized in 2004 were Gram-negative species such as Actinobacillus actinomycetemcomitans (currently *Aggregatibacter actinomycetemcomitans*), Porphyromonas gingivalis, Bacteroides forsythus (currently Tannerella forsythia) and Eikenella corrodens (Preshaw et al., 2004).

The concepts of periodontal disease from 2004 were recently updated by Kwon, T. et al., 2021, who recognized that the prevalence of periodontal disease increased from 15% to more than 40% in American adults. The authors also note the multifactorial etiology of periodontal disease, including subgingival dental biofilm (Kwon et al., 20121). The disease pathogenesis is presented in Figure I.2.12.

Bringuier, A. et al. (2013) detected Methanobrevibacter oralis by qRT PCR in both periodontal patients and age-matched controls, at similar proportions (100% vs. 80%). Due to methanogen-targeting molecular investigations, the oral cavity inhabitant, Methanobrevibacter oralis, was found to be more strongly associated with periodontitis pockets in association with anaerobes, and its role was analyzed as moderate (Belkacemi et al., 2018).

A comprehensive, recent review presents the roles of normal flora from the oral cavity, and mentions its function in oral mucosa homeostasis and in stimulating the immune system, especially in the case of periodontitis (Ptasiewicz et al., 2022).

Periodontal disease management requires standardization in the diagnosis and reporting of chronic periodontitis. A multidisciplinary team underlined the need to note the study design (e.g.,

inclusion criteria for participants, regional versus national study, type of sampling, sample size), assess periodontal measurements and record the protocols (e.g., analyzing periodontal pockets, probing pocket depth, a full-mouth recording), the need for a periodontal probe (inter-and intra-examiner variability is very important), and the importance of ensuring the examiners' reliability.

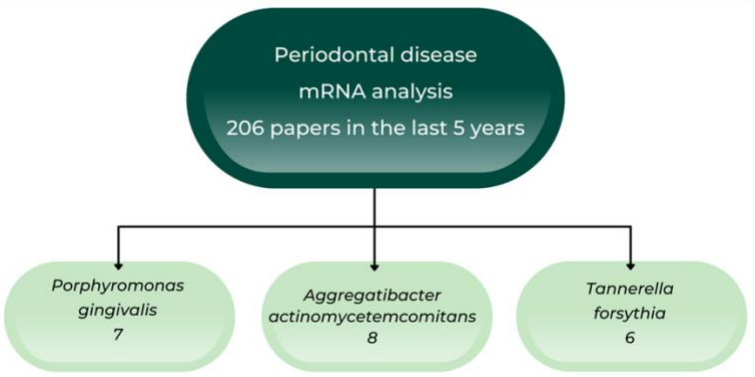


Figure I.2.12. Diagram of analyzed studies.

The authors referred to the manner of reporting periodontal studies regarding the characteristics of study subjects, and the reporting of the prevalence and severity of periodontal diseases in accordance with periodontal case definitions and gingival inflammation. All these determinants of periodontitis prevalence and severity can optimize the control of the burden of periodontitis worldwide. By using standardized protocols when reporting each case of periodontal disease, variations between different populations will be eliminated (Holtfreter et al., 2015).

In an observational study, another international research team (Germany, Hong Kong, and Spain) underlined the need for standardization in periodontal disease screening. The authors compared specific databases and found that bleeding on probing has the strongest association with severe periodontitis. They developed an easy-to-use guide for daily practice (Adel-Khatta et al., 2021).

As in other medical fields, in periodontal disease, Swedish researchers implemented a means of monitoring dental health and healthcare by registering data about patients (gender, age, living area, dental status, risk assessments for caries and periodontitis and dental care provided). This systemic registration of oral health and quality of dental care will facilitate clinical and epidemiological research and randomized controlled trials (von Bültzingslöwen et al., 2019). In Sweden, a research team performed a longitudinal study regarding indicators of periodontitis, such as alveolar bone loss, for ten years in an older population.

The authors used a questionnaire and performed a clinical examination. Alveolar bone loss was associated with poor general health and irregularly undergoing dental care (Edman et al., 2022). Periodontal disease was found to be associated with other pathologies, such as rheumatoid arthritis (Renvert et al., 2020) and asymptomatic carotid plaque (Jonsson et al., 2020).

Although the main scientific databases contain many recent papers regarding the identification of periodontal pathogens, we only found 206 articles published in the last five years referring to the importance of mRNA analysis in periodontal diagnosis (Figure I.2.12).

The aim of this review was to analyze the scientific literature published in the last five years regarding the recent applications of mRNA analysis to periodontal disease for the main known bacterial species considered to be the etiological agents: Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Tannerella forsythia. We referred to circular RNAs in periodontal disease, and to the actual issues and controversies regarding the optimal therapy of periodontal diseases.

Updates in Periodontal Disease Classification

In 2018, a consensus report was published on the classification of periodontal and peri-implant diseases and conditions. This update was necessary because, in recent years, the classification of periodontitis has been repeatedly modified in an extremely important attempt to align it with the newest scientific evidence (Caton et al., 2018).

In order to provide a properly updated version of the previous classification of Armitage, it was mandatory for the members of the study group to redefine the state of periodontal and gingival health (Figure I.2.13.).

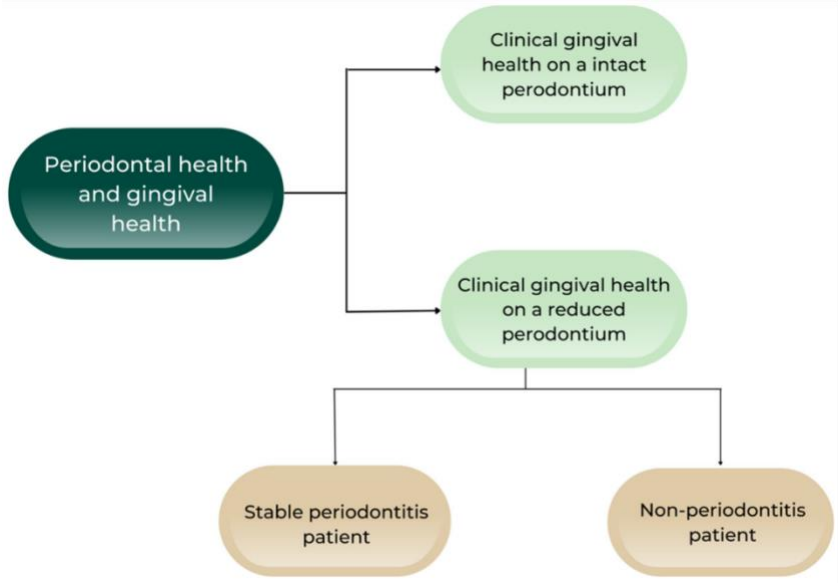


Figure I.2.13. Periodontal and gingival health (Caton et al., 2018)

Jepsen et al., 2018, provided an updated classification of the periodontal manifestations and conditions affecting the course of periodontitis and the periodontal attachment apparatus, as well as its development and acquired conditions. The authors presented the systemic diseases and conditions that affect the periodontal supporting tissues, e.g., diseases associated with immunologic disorders such as Down syndrome, acquired immunodeficiency diseases (e.g., acquired neutropenia), inflammatory diseases such as Epidermolysis bullosa acquisita, other systemic disorders that influence the pathogenesis of periodontal diseases, such as diabetes

mellitus, and neoplasms that can result in the loss of periodontal tissues independent of periodontitis. Mucogingival conditions related to natural dentition referred to possible consequences of gingival recession and root surface exposure to oral environment, the development of gingival recession associated with the gingival phenotype, periodontal phenotype assessments in a standardized and reproducible way and the classification of gingival recession (Jepsen et al., 2018).

The current classification introduced a much more accurate and predictable approach to the diagnosis of periodontitis, using stages and grades and referring to the extent and distribution. Stages are based on the severity and complexity of management, while grades refer to evidence or risk or progression in the context of anticipation of the treatment response (Figure I.2.14.).

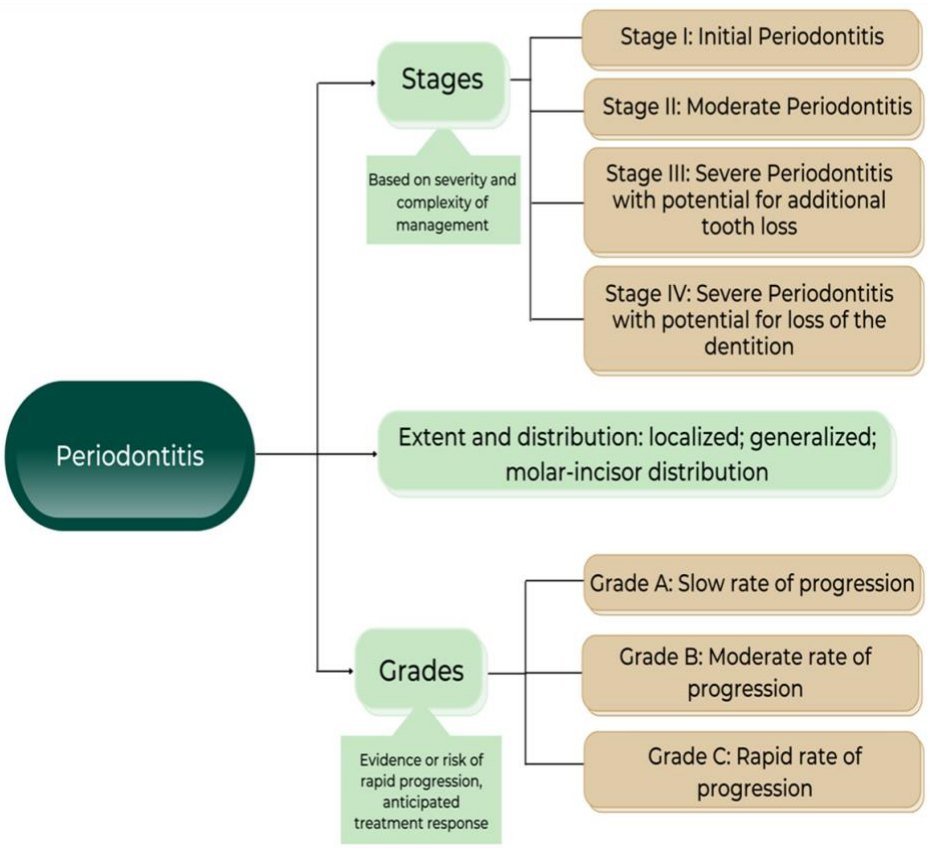


Figure I.2.14. Classification of periodontitis (Caton et al., 2018).

The recently published clinical practice guidelines for the treatment of periodontitis in stages I–III provided evidence-based recommendations for the treatment of periodontitis patients, defined according to the 2018 classification (Sanz et al., 2020). Stage IV periodontitis was recently updated to maintain a healthy dentition over one's lifetime (Herrera et al., 2022).

1.7.2. Biomarkers early detection by mRNA assays

MicroRNA was reviewed by Catalanotto C et al. and the authors mentioned its influence on many physiological processes, such as differentiation, proliferation, apoptosis and development, its cytoplasmatic functions, nuclear functions and host cell's miRNAs, which target viral mRNAs (Catalanotto et al., 2016). To analyze the RNA, there is a need for cutting-edge technologies in the field of molecular biology to implement novel biotechnological and medical applications of RNA, such as, for example, in regenerative medicine to promote stronger cardiovascular outcomes (Matta et al., 2022).

Several mRNA methods have been used in the management of other diseases, such as in the case of non-small lung cell carcinoma, where a research team used a large array of such investigations: Western Blot analysis and antibodies detection, protein extracts from human tissue samples, cell Cultures, siRNA and DNA transfections, plasmids and cloning strategies, total mRNA extracts' purification, RT-qPCR and RT-ddPCR analysis, RNA immunoprecipitation and RNA chromatography assays, migration, invasion and proliferation assays, flow-cytometry analysis, epifluorescence microscopy and immunohistochemistry (Bonnet-Magnaval et al., 2021)

circRNAs exhibit specific characteristics, making them ideal biomarkers for diagnosis and prognosis.

Mi Z et al. (2022) mentioned in a review that traditional methods, such as northern blotting, RT-qPCR and microarray analysis, provide useful but limited information. New techniques are available for circRNA detection, such as RT-ddPCR, RCA and LAMP, with their own advantages and limitations (Mi et al., 2022). A recent review (September 2022) mentions the latest developments in the analysis of nucleic acids using capillary electrophoresis and its applications for ASO, siRNA, mRNA, gRNA, microRNA, AAV and aptamers. Each of these therapeutic nucleic acid analyses should be tested in terms of analytical challenges and future perspectives (Wei et al., 2022).

In Table I.2.6., the recent findings in periodontal disease using mRNA analysis for Porphyromonas gingivalis are presented. The studies were performed from Finland to China; the authors used samples from patients (e.g., gingival biopsies) and studied different in vitro models (e.g., cell lines). The findings were especially correlated with the identification of different molecules by mRNA, which could elucidate the pathogenesis of periodontal disease: caspase-4 activation in P. gingivalis-infected gingival epithelial cells (GECs), monocyte chemoattractant protein-1-induced protein (MCPIP-1) and mucosa-associated lymphoid tissue lymphoma translocation protein (MALT-1) responses, the target gene MZB1, CTHRC1. Type IX protein secretion system (T9SS) shutdown was found to influence the inflammatory response in periodontal pathogens and is also considered a potential novel target for periodontal therapy.

The mRNA analysis regarding *Tannerella forsythia* mainly tried to identify pathogenic mechanisms (KLIKK-proteases, cytokine IL-1α levels, NLRP3 and AIM2 proteins, and TREM-1 (triggering receptor expressed on myeloid cells 1 tissue expression) and preventive actions (Litsea japonica leaf extract) (Table I.2.7.)

Table I.2.6. Associations between periodontal disease and *Porphyromonas gingivalis* by mRNA analysis.

Authors Year, Country	Sample Type	mRNA Analysis Assay	Results	Novelty
Kantrong N et al., 2022, Khon Kaen, Thailand (Kantrong et al., 2022)	Gingival biopsies, healthy participants with periodontitis or clinically healthy gingiva	mRNA expressions/RT-qPCR of human β-defensin-2 (hBD-2), interleukin (IL-) 8, and IL-18 in stimulated GECs in the presence or absence of a caspase-4 inhibitor	mRNA upregulations of hBD-2, IL-8, and IL-18 upon *P. gingivalis* stimulation were significantly reduced by caspase-4 inhibition ($p < 0.05$), while, for *F. nucleatum*, the inhibitor did not exhibit the same suppressor activity	Caspase-4 activation in *P. gingivalis*-infected GECs showed an upregulation of immune effector molecules, suggesting a possible detection mechanism of caspase-4 in GECs in periodontal disease pathogenesis
Firatli Y et al., 2022, Turku, Finland (Firatli et al., 2022)	Human gingival keratinocyte (HMK) monolayers were incubated with *P. gingivalis*, *F. nucleatum*, *P. gingivalis* LPS and IL-1β.	Immunoblots and mRNA levels by qPCR for protein levels of MCPIP-1 and MALT-1	MCPIP-1 mRNA levels were increased by *P. gingivalis*, *F. nucleatum*, and IL-1β, but no change was detected in MALT-1 mRNA levels	- Infection and inflammatory mediators regulate the gingival keratinocyte MCPIP-1 and MALT-1 mRNA and protein expression responses. - Periodontitis-associated bacteria-induced modifications in MCPIP-1 and MALT-1 responses can be a part of periodontal disease pathogenesis
Li D et al., 2022, Chongqing, China (Li et al., 2022)	human gingival tissues	Dual-luciferase reporter assay, which assessed the binding of miR-185-5p to MZB1 (ER-localized protein)	MZB1 was markedly increased in the gingival tissues of periodontitis patients, in mouse models, and in the hPDLCs treated with lipopolysaccharide of *P. gingivalis* (LPS-PG)	MZB1 (a target gene of miR-185-5p) plays an important role in inhibiting the migration of hPDLCs through NF-κB signaling pathway and deteriorating alveolar bone loss
Wang H wr al., 2022, Shenyang, Liaoning Province, China	the effects of the *P. gingivalis* outer membrane protein OmpH (encoded by PG0192 and PG0193) on IL-6 and tumor necrosis factor-α (TNF-α) expression in macrophages to assess the pro-inflammatory cytokine responses	Macrophages treated with mutant strains (PG0192-PG0193 deletion) showed a downregulation in the expression of IL-6 and TNF-α at mRNA and protein levels	IL-6 and TNF-α mRNA levels were up-regulated following treatment of macrophages with *P. gingivalis* W83 co-incubated with rOmpH-1 or rOmpH-2	- The roles of PG0192 and PG0193 in promoting IL-6 and TNF-α expression in macrophages exposed to *P. gingivalis* - Involvement of C5aR in the pro-inflammatory response
Bekić M et al., 2022, Belgrade, Serbia	10 H-GMSC and 12 P-GMSC lines	*P. gingivalis* up-regulated the mRNA expression of IL-6, IL-8, MCP-1, GRO-α, RANTES, TLR-2, HIF-1α, OPG, MMP-3, SDF-1, HGF and IP-10 in P-GMSCs, while only IL-6, MCP-1 and GRO-α were up-regulated in H-GMSCs.	P-GMSCs had a significantly higher expression of MCP-1, RANTES, IP-10 and HGF compared to H-GMSCs, but IDO1 was lower	Cultures of P-GMSCs retain their pro-inflammatory properties, while exhibiting lower immunosuppressive potential than their healthy counterparts, and reduced regeneration-associated gene induction in culture. All these functions are positively influenced by *P. gingivalis* treatment
Huang XY et al., 2022, Fuzhou, China	gingival tissue samples from clinically healthy subjects (15 cases) and patients with periodontitis (30 cases)	mRNA levels of the intracellular collagen triple helix repeat containing-1 (CTHRC1) and protein expression of the extracellular CTHRC1	In the periodontitis group, protein expression of CTHRC1 was higher than that of the clinically healthy group	- CTHRC1 might play a role in the development of periodontitis - Expression level might be significantly correlated with the stimulation produced by *P. gingivalis* LPS on fibroblasts
Braun ML et al., 2022, Vienna, Austria	wild-type *T. forsythia* and *P. gingivalis* and T9SS signal peptidase-deficient mutants defective in protein secretion were used to stimulate human macrophages and gingival fibroblasts	mRNA expression levels of the pro-inflammatory mediators IL-6, IL-8, MCP-1 and TNF-α by qPCR	- 16 h post-stimulation, the *T. forsythia* T9SS mutant induced a significantly lower production of cytokines and the chemokine in cells compared to the corresponding wild-type strain - The opposite was noted for the *P. gingivalis* T9SS mutant	- T9SS shut-down translates into an altered inflammatory response towards periodontal pathogens - T9SS needs further evaluation as a potential novel target for periodontal therapy

Table I.2.7. Associations between periodontal disease and *Tannerella forsythia* by mRNA analysis.

Authors Year, Country	Sample Type	mRNA Analysis Assay	Results	Novelty
Braun ML et al., 2022, Vienna, Austria	Wild-type *T. forsythia* and *P. gingivalis*and T9SS signal peptidase-deficient mutants defective in protein secretion were used to stimulate human macrophages and gingival fibroblasts	mRNA expression levels of the pro-inflammatory mediators IL-6, IL-8, MCP-1 and TNF-α by qPCR	T9SS shutdown translates into an altered inflammatory response in periodontal pathogens	T9SS as a potential novel target for periodontal therapy needs further evaluation
Yun IG et al., 2018, Gwangju, Korea	The ability of *Litsea japonica* leaf extract (LJLE) to inhibit pro-inflammatory cytokine production in PDLFs in response to various stimulants	mRNA and protein expression	Anti-inflammatory effect of LJLE in PDLFs after infection with various oral bacteria, including *F. nucleatum*, *P. gingivalis*, *Treponema denticola*, and *T. forsythia*	LJLE has anti-inflammatory activity that could be exploited to control inflammation in human periodontitis
Eckert M et al., 2018, Bern, Switzerland	Biofilm and gingival crevicular fluid (GCF)/ peri-implant sulcular fluid (PISF) samples were taken from 10 healthy tooth and implant sites, 12 gingivitis and mucositis sites, and 10 periodontitis and peri-implantitis sites	mRNA expression of individual genes	Gingipains' expression level was associated with the levels of miropin and certain *T. forsythia*proteases around teeth but not implants	KLIKK-proteases, especially miropin, might be involved in the pathogenesis of both periodontal and peri-implant diseases
Lee SJ et al., 2017, Seoul, Korea	The human oral epithelial cell line HOK-16B was infected *T. forsythia* and *F. nucleatum*, at various MOIs	RT-PCR and immunoblotting assays for mRNA and protein expression	Infection increased mRNA and protein expression of NLRP10, respectively NLRP10 is involved in activating the ERK signaling pathway in HOK-16B cells infected with *T. forsythia* and *F. nucleatum*	Pro-inflammatory cytokine IL-1α levels are augmented by the activation of the ERK pathway, which may play a critical role in periodontitis
Ran S et al., 2017, Shanghai, China	Periapical lesions	mRNA levels of apoptosis-associated speck-like protein (ASC), caspase-1IL-1β, NLRP3 and AIM2 in THP-1-derived macrophages treated with *Porphyromonas* LPS were quantified by real-time PCR	Up-regulation of NLRP3 mRNA was correlated with a simultaneous up-regulation of caspase-1 mRNA in most samples	NLRP3 and AIM2 proteins are involved in the pathogenesis of periapical periodontitis. Anaerobes, such as *P. endodontalis*, *P. gingivalis*, *F. nucleatum* and *T. forsythia*, were the most important microbial stimuli that might activate inflammasomes in periapical tissues
Willi M et al., 2014, Zürich, Switzerland	Gingival tissue	TREM-1 mRNA expression	TREM-1 expression was found to be increased in both aggressive and chronic periodontitis, compared to healthy tissues, and correlated with the levels of the 'red complex' species in the tissue	TREM-1 tissue expression is up-regulated in periodontal disease and correlates with the level of periodontal pathogens

Oral Anaerobic Bacteria and Cancer.

Prevotella, *Fusobacterium*, *Porphyromonas*, *Treponema* and *Aggregatibacter* genera were associated with periodontal disease in a recent review (Ptasiewicz et al., 2022).

While the most important and well-studied human pathogens associated with cancers are viruses (Ursu et al., 2021) in recent years, a link has begun to appear between anaerobic bacteria found in the oral cavity and tumors of the gastrointestinal tract, especially colorectal carcinoma, with the most important representative being Fusobacterium nucleatum. This is a Gram-negative, anaerobic bacteria that can be found as part of the oral microbiome, and when dysbiosis occurs in association with other bacteria, it produces gingivitis and periodontal disease. F. nucleatum seems to tend to disseminate from the oral cavity to other sites of the human body, probably via hematogenous transfer, and is frequently found in placental and fetal tissues, especially in adverse pregnancy outcomes (Han 2015).

While abundant in the oral microbiota, this bacterium is seldom found in the healthy colon, although numerous studies have shown an increased presence of F. nucleatum in colorectal cancer samples. Castellarin et al., as well as Kostic et al., found increased levels of fusobacterial nucleic acids in colorectal carcinoma samples in 2012, while the same increase was not present in adjacent normal tissues. Since F. nucleatum is not normally present in high amounts in the lower GI tract, it has been proposed that the presence of this bacteria is used in colorectal carcinoma screening, diagnosis and disease follow-up.

In a meta-analysis study, Zhang et al. found that testing for F. nucleatum alone in fecal samples has a pooled sensitivity and specificity of 71 and 76%, respectively, making it a valuable tool in the diagnosis of such tumors (Zhang et al., 2019). Guo et al. compared the ratios of F. nucleatum to other bacteria normally present in fecal samples (Faecalibacterium prausnitzii, Bifidobacterium spp., Lactobacillus) in two cohorts composed of 903 patients, and found the F. nucleatum/Bifidobacterium ratio to have an 84.6% specificity and 92.3% sensitivity in detecting colorectal carcinoma. Combining F. nucleatum/Bifidobacterium with F. nucleatum/F. prausnitzii assays could be an efficient, noninvasive screening test, able to detect stage I colorectal carcinoma with 60% specificity and 90% sensitivity (Guo et al., 2018).

Other authors proposed wider detection panels, using *Parvimonas micra*, *Peptostreptococcus stomatis*, *Fusobacterium nucleatum* and *Akkermansia muciniphila* as cancer biomarkers (Osman et al., 2021).

The specific target used by F. nucleatum to recognize and adhere to tumoral cells is the molecule d-galactose-β(1-3)-N-acetyl-d-galactosamine, commonly known as Gal/GalNAc, which is overly abundant in colorectal carcinoma. The specific ligand for Gal/GalNAc seems to be the fusobacterial adhesin Fap2 (Ghosh et al., 2016), an important virulence factor of F. nucleatum, especially for co-aggregation, together with other oral anaerobes such as Porphyromonas gingivalis in the pathogenic mechanism of periodontitis (Coppenhagen-Glazer et al., 2015).

Placental tissues are also rich in Gal/GalNAc; thus, the mechanism of F. nucleatum colonization of the placenta in adverse pregnancy outcomes must be due to the same Gal/GaNAc-Fap2 interaction (Parhi et al., 2022).

Another type of tumor that is particularly abundant in Gal/GalNAc is breast cancer, and the level of this marker increases with tumoral progression, a discovery that can now explain the high prevalence of F. nucleatum in the breast cancer microbiome (Parhi et al., 2020).

Experimental studies in mouse models showed that, in addition to colonizing the tumoral tissues, F. nucleatum has negative effects on disease progression and metastatic development, inducing the suppression of T-cell numbers in the tumor. By intravenously inoculating one group of mice with F. nucleatum capable of expressing Fap2, and another group with a Fap2-deficient strain, the authors showed that Fap2 is vital for tumor colonization, and that tumors colonized with these anaerobic bacteria had an increased size and number of metastases (Parhi et al., 2020).

Regarding therapy outcomes and survival, Kunzmann et al. found that although high F. nucleatum DNA levels in colorectal tumors are associated with poorer survival outcomes, their study indicated that this assay has limited clinical use for predicting prognosis (Kunzmann et al., 2019). In esophageal carcinoma, some authors found that high levels of intratumoral F. nucleatum are significant when predicting a poorer response to neoadjuvant chemotherapy and suggest that antibiotic therapy could improve outcomes (Yamamura et al., 2019).

While some authors focus on antibiotics as a solution to combat periodontitis pathogens in the oral cavity (Luchian et al., 2021), some novel therapeutic strategies have also been proposed in studies that link those pathogens to cancer. One such strategy, which exploits the association between Fusobacterium nucleatum and colorectal carcinoma, has been studied by Zheng et al. 2019, using a nanotechnological microbiome-modulating intervention. The research team used a bacteriophage that specifically targets *F. nucleatum* to reach tumoral tissues and lyse these bacteria, reducing their pro-tumoral effects.

1.7.3. Circular RNAs Assessing in Periodontal Disease

Circular RNAs (circRNAs) can influence disease progression by targeting miRNA/mRNA axis. Periodontal disease was intensely studied in connection with this biomarker. Deng W et al. used different assays (qRT-PCR, cell proliferation, wound healing, cell apoptosis, enzyme-linked immunosorbent assay (ELISA)) on periodontitis cell models and identified that circ_0138959 was overexpressed in periodontitis tissues and LPS-treated periodontal ligament cells, which could be a suitable target for periodontal disease therapy (Deng et al., 2022).

Another possible therapeutic target for periodontal disease is circ_0062491, which was found by Wang L et al. to protect PDLCs from LPS-induced apoptosis and inflammation. In this study, the authors used cell counting Kit-8 (CCK-8) assay, flow cytometry and Western blot, in addition to the above-mentioned techniques (Wang et al., 2022).

Using the previously mentioned assays, together with the dual-luciferase reporter assay and RNA immunoprecipitation assay for validation of target interaction, Li Q et al. showed that circ_0066881 partly prevented LPS-evoked cell dysfunction in PDLCs through the miR-144-5p-mediated up-regulation of retinoid acid-related orphan receptor A (Li et al., 2022). Using high-throughput sequencing and qRT-PCR, Yu W et al. identified differentially expressed circRNAs in gingival tissues from periodontitis patients, as it is known that periodontal disease is a chronic

multifactorial inflammatory disease. The authors detected 70 differentially expressed circRNAs (68 up-regulated and 2 down-regulated circRNAs) in human periodontitis, and they found a positive correlation between up-regulated circRNAs, circPTP4A2, chr22:23101560-23135351+, circARHGEF28, circBARD1 and circRASA2, and the PD-suggested function of circRNAs in periodontitis (Yu et al., 2022).

A very interesting study analyzed the circRNAs in periodontal tissues in patients with or without Redondoviridae-infection—DNA viruses known to be associated with periodontitis. The authors used a high-throughput RNA sequencing assay to understand the pathogenetic mechanisms of the Redondoviridae-related periodontitis, to see if it is possible to use these viruses as biomarkers and, in future, targeted therapies (Zhang et al., 2022).

Circular RNAs' role in periodontal disease has also been studied by other authors, with the main findings being the overexpression of hsa_circ_0003948 with a protective effect in chronic periodontitis via miR-144-3p/NR2F2/PTEN signaling regulation (Li et al., 2022), and a promising biomarker for periodontitis treatment, circ_0085289, alleviated PDLC injury induced by LPS stimulation by modulating the let-7f-5p/SOCS6 axis (Du et al., 2021).

circRNAs are starting to have more applications in periodontal disease, as they can be used to accurately diagnose periodontitis activity: circRNAs are expressed in periodontal cells in a cell-specific manner, can function as microRNA sponges and can form circRNA-miRNA–mRNA networks during osteogenic differentiation for periodontal-tissue (or dental pulp)-derived progenitor cells (Jiao et al., 2021). The above-mentioned studies underline the opportunity revealed by RNA analysis in periodontal diseases, which could lead to an understanding of the pathogenetic mechanisms and to new targeted therapies.

RNA-seq and Periodontal Diseases.

Teles F et al. 2021, recognized the utility of RNA sequencing in periodontal disease, in a recent review. The authors found new NGS findings regarding the relationship between periodontal disease and systemic factors, with benefits for the patient for follow-up and therapy.

There are many published studies regarding periodontal disease and RNA sequencing analysis. Chen X et al. used 16S rRNA gene sequencing analyses for patients with Crohn's disease and found that both red complex (Porphyromonas, Tannerella and Treponema) and orange complex (Fusobacteria) bacteria were abundant in periodontitis subgingival plaque, in comparison with orange complex bacteria (Prevotella_2 and Prevotella), which was overexpressed in Crohn's disease-associated periodontitis subgingival plaque. The authors recognize the advantage of using 16S rRNA to reveal the oral microbiome in CD-associated periodontitis in comparison with periodontal patients without this condition (Chen et al., 2022).

Ge D et al. used the 16S rRNA sequence of P. gingivalis for studying patients with periodontal disease. The recombinase polymerase amplification, combined with nanoparticle-based lateral flow strips for the rapid detection of P. gingivalis, was found to be an efficient, rapid (30 min) and convenient diagnostic method that optimizes the classical diagnosis of detecting P. gingivalis (Ge et al., 2022).

In a recent meta-analysis, Jiang Y et al., 2021, made a comparison between saliva and subgingival plaque using 16S rRNA gene sequencing techniques. They revealed that both the detection frequencies and relative abundances of red-complex bacteria in saliva were significantly lower than those in subgingival plaque, leading to the conclusion that there is a need for further longitudinal clinical studies to evaluate the role of saliva.

Using 16S rRNA amplicon sequencing, Chang C et al. (2019) analyzed the relationship between periodontal pathogens and oral squamous cell carcinoma (OSCC). The researchers identified that P. gingivalis and F. nucleatum were present at higher levels in cancer tissue than in normal tissues and were correlated with subgingival plaques. This raised awareness regarding the involvement of the above periodontal pathogens and OSCC, in addition to the known risk factors, such as HPV, smoking and chronic alcohol use.

Smoking, as a risk factor altering salivary microbiomes, was analyzed in a prospective study using sequencing of 16S recombinant RNA gene amplicons. It is important to mention that Porphyromonas gingivalis was significantly more abundant in smokers, which suggests that smoking could influence the salivary microbiome and affect marginal bone loss during bone healing (Duan et al., 2017).

The microbial 16S rRNA gene sequencing was performed by Lundmark A. et al. to assess whether salivary microbiota is associated with host inflammatory mediators in periodontitis. The Swedish authors identified distinct and disease-specific patterns of salivary microbial composition between patients with periodontitis and healthy controls, noting that Tannerella forsythia was more frequently present in periodontitis (Lundmark et al., 2019).

Moreno C et al. (2022) conducted a meta-analysis that included two independent RNA-seq datasets to identify diagnostic biomarkers and specific pathways for a new targeted periodontitis therapy, such as chronic inflammation. The authors compiled a list of the top 10 drugs that should be further tested for their efficacy in treating periodontitis.All the above-mentioned studies underline the clinical utility of RNA-seq in different clinical conditions associated with periodontal diseases.

7. The Optimal Therapy of Periodontal Diseases

We analyzed recent randomized controlled trials regarding the antibiotic therapy of periodontal disease.

Blanco C et al. (2022) studied the clinical, radiographic and microbiological outcomes after non-surgical therapy of peri-implantitis for 32 patients, followed up for a period of 12 months. The authors considered that metronidazole as a systemic therapy led to significant additional improvements in clinical, radiographic and microbiological parameters.

Teles et al. (2021) studied the percentage and taxonomy of minocycline-resistant isolates in saliva and subgingival plaque samples before and after minocycline microspheres application in periodontitis patients during maintenance.

The patients were monitored for 6 months, and the authors found that even Aggregatibacter actinomycetemcomitans, Tannerella forsythia and Porphyromonas gingivalis were sensitive to antibiotics, and minocycline microspheres resulted in the transient selection of

minocycline resistant species in saliva and subgingival plaque samples, such as Gemella morbillorum and Eubacterium saburreum.

Cosgarea et al. (2020) used the antibiotic protocol (amoxicillin (AMX) + metronidazole (MET)) for 102 patients for 12-month follow-up in nonsurgical periodontal therapy to obtain the maximum antimicrobial benefit and minimum risk for adverse effects. After comprehensive monitoring of the patients (ELISA and RT PCR for the detection of etiologic agents and inflammatory markers), the authors concluded that (AMX + MET) antibiotic protocols led to greater microbiological improvements compared to subgingival debridement alone.

Cha et al.(2019) also studied the clinical, microbial and radiographic effects of local minocycline combined with surgical treatment of peri-implantitis. The authors found that the repeated local delivery of minocycline combined with surgical treatment provides significant benefits in terms of clinical parameters and radiographic bone fill.

Luchian I et al. (2021) mentioned in a review that clindamycin offers several advantages for periodontal treatment, both systemically and locally, with various degrees of success, such as the enhancement of neutrophil chemotaxis, phagocytosis and the oxidative burst-oxidative stress storm, which are easily absorbed at the level of oral tissues in a considerable amount, substantial tissue penetration, especially inside the bone. All the above-mentioned features are synergistic, with a stimulating effect on the host immune system.

The management of periodontal diseases is an important issue. The American Dental Association Council on Scientific Affairs and the Center for Evidence-Based Dentistry conducted a systematic review and formulated clinical recommendations.

The panel recommended against using antibiotics in most clinical scenarios, irrespective of definitive, conservative dental treatment availability. The experts suggested that antibiotics for target conditions should only be used when systemic involvement is present to avoid all the side effects of antibiotic therapy (Lockhart et al., 2019).

1.7.4. Final remarks

Periodontal disease is one of the most prevalent pathologies worldwide and it has the great advantage of being detectable in an early and efficient manner through RNA methods.

Using mRNA and circRNAs technologies, it was possible to identify new pathogenic mechanisms, new target genes and protective compounds, which lead to an improvement in the prognostic and may optimize future therapeutic protocols. Therefore, RNA-based techniques can successfully detect periodontal bacteria much more accurately than others and they might represent a “state-of-the-art” diagnostic tool in the future.

The technology used for mRNA analysis should be standardized in the near future to be safely used by many clinicians in cooperation with molecular biology specialists as a useful tool for the early diagnosis of periodontitis.

CHAPTER 3:
BACK TO THE FUTURE. ALTERNATIVE OPTIONS FOR ENHANCING PERIODONTAL THERAPY

1.8. State of the art

Williams (1852 – 1932) first described dental plaque as "gelatinous accumulation of the bacteria adherent to the enamel surface. In the early 1880s, common crown and bridge techniques were not well known or widespread. Williams sought to make information on these techniques available to all dentists through the pages of The Dental Cosmos. In 1885, he embarked on a journey to improve the state of dental prostheses by designing more aesthetic artificial teeth that better matched the overall facial dimensions of the patient. He convinced an American artificial tooth manufacturer to take up his cause and produce his designs, on the condition that other dentists would embrace the new forms (Clapp, 1932).

Comparative longitudinal studies, surgical versus nonsurgical, demonstrated that both surgical and nonsurgical therapy result in limited regeneration and healing with a long junctional epithelium. The most important aspects of today's modern concept of periodontal therapy are causal, regenerative, and specific for disease type and severity. Although the regeneration of the periodontium can be accomplished with the biological principles of guided tissue regeneration and graft materials, compared to conventional methods, the restoration of a completely normal periodontal status has not yet been achieved. We are about to reach our ultimate goals and presently, the more promising research directions for a substantial regeneration seems to lie in biological mediators. Although the future of periodontal therapy is bright, it is still of critical importance to have a preventive strategy to keep individuals healthy beforehand (Yilmaz et al., 1994).

Periodontal disease has been seen in skeletal remains since before recorded history. Anthropologic expeditions to Mesopotamian sites in Iraq found relics used for dental care in gold vanity sets and cases, including ear scoops, tweezers, and toothpicks. One set found in the Nigel Temple at Ur is estimated to have been used about 3000 BCE.

The oldest written documents related to teeth are from about the same era and were found in the excavations of the Sumerian civilization in the Middle East. These are pictographic and cuneiform tablets and contain descriptions such as this: "If a man's mouth has mouth trouble, thou shalt bray [grind finely] Lelium in well water, introduce salt, alum and vinegar therein, thou shalt leave it under the stars, in the morning, thou shalt wind a linen [strip] around his forefinger, without a meal thou shalt clean his mouth." (Patey, 2013).

Hippocrates (460-377 BCE), a Greek physician, was the first person known to prescribe a dentifrice. His cure was not something we would use today because it was a complex solution, including the head of a hare and three mice to be sieved and soaked in honey and white wine and then rubbed on the gums. A twentieth century study of periodontoclasia, an early term for any destructive or degenerative disease of the periodontium, suggested that Hippocrates may have

provided relatively effective periodontal treatment long before modern times. Aristotle, who was born after Hippocrates (348-322 BCE), described "scrapers" used for teeth cleaning. Surviving examples show that these instruments were similar to modern scalers. Interestingly, Aristotle did not think that women had as many teeth as men (Elibasakis, 2023).

Fiber sticks were the earliest known toothbrushes. They were the size of pencils and were hammered or flattened on one end to separate the fibers. Such sticks were recorded in Babylonian, Chinese, Greek, and Roman literature. All were made from trees or bushes with bark containing a cleansing substance plus aromatic fumes that acted as an astringent, like a kind of dentifrice. Early Mohammedans called their sticks "siwaks," which were made of arrak (Salvadora persica, the toothbrush tree). In Greece and Italy, sticks came from the mastic tree (Pistacia lentiscus, the toothpick tree). In Saudi Arabia, the sticks are called "miswaks" (Löe et al.,1978).

The era of modern dentistry began in Europe. Dentists were trained by apprenticeship, learning by watching and assisting an established dentist. Ambroise Paré was the first apprentice permitted to take an examination and apply for membership in the prestigious College of Surgeons in Paris, a privilege reserved for physicians. He extracted teeth, opened dental abscesses, set fractured jaws, and was recognized by medical colleagues for his professional work (Andrew 2004).

Andreas Vesalius and Bartolommeo Eustachio were responsible for early anatomic studies of the teeth. In the following century, Anton van Leeuwenhoek discovered dentinal tubules when looking through his invention, the microscope. He also examined tartar scrapings from teeth and identified microorganisms in the mouth.

Descriptions of early attempts at treating them were recorded, but in general, periodontal diseases were assumed to be incurable. In 1845 John M. Riggs first publicly called attention to this disease in the United States. He asserted that it was a curable disease and that with proper surgical treatment (by this he meant cleaning of the periodontal pockets), 90% of the cases could be cured. His opinion became widely accepted and periodontal disease became known as Riggs' disease. Curettage beyond the confines of the periodontal pocket to "stir up a healing reaction" was his original contribution to therapy (Hall 1977).

Leonard Koecker, a nineteenth century surgeon-dentist in London, was acknowledged to have successfully treated advanced cases of periodontal disease with conservative techniques. He is considered the first periodontist. His treatment to cure periodontoclasia, as it was called at the time, was very controversial. However, Riggs adopted these techniques and demonstrated them in public clinics. When dentists saw the results, these treatments became accepted (Koecker 1850).

F.H. Rehwinkel renamed periodontoclasia pyorrhea alveolaris in a report he presented to the American Dental Association (ADA) in 1877. This name was never totally accepted because it was descriptive of only one aspect of pathology, bone loss. However, pyorrhea became a commonly used term that is still used today (Weinrich et al., 1986).

Late in the 1800s, dental journals began to include articles about disease prevention and patient education. D.D. Smith of Philadelphia emphasized systematic change in the oral environment surrounding the teeth to prevent disease and demonstrated these techniques to colleagues. Smith had a profound influence on Alfred C. Fones, the founder of the role of the dental hygienist. Fones attended a meeting of the Northeastern Dental Society in 1898 at which Smith described his system of periodic oral prophylaxis (cleaning of the teeth). The treatment required patients to return at intervals of a few weeks for office treatment and to perform daily home care as they were instructed. Fones visited Smith's office three times to observe the practice. He then implemented this system in his own office for 5 years.

Fones recognized that the procedures took an inordinate amount of his practice time. Smith believed that prophylaxis was too important to be delegated, but Fones disagreed and sought to develop an assistant to provide this care. Esthetics is one of the most frequent reasons why patients seek consultation in the areas of implant dentistry and periodontics (Magne et al., 1994).

Clinicians have become concerned about the management of peri-implant and periodontal hard and soft tissue. The esthetic zone is defined as the area delimited by the lip perimeter. Restorative and esthetic dentistry requires a comprehensive approach: the first step of any treatment plan must be basic therapy (De Jesús Tavarez et al., 2012).

Periodontal plastic surgery is defined as the plastic surgical procedures designed to correct defects in morphology, position, and/or amount of gingiva surrounding the teeth. This definition also applies to peri-implant tissue management. Some indications: esthetic concerns, cases where dental plaque is difficult to control in the recession area, prior to orthodontic treatment in cases where movement can entail risk of recession, and prior to rehabilitation in areas without attached gingiva. We now have the concept of evidence based periodontal plastic surgery, which is defined as the "systematic evaluation of clinically significant scientific evidence intended to investigate the esthetic and functional effects of treatment of defects of the gingiva, alveolar mucosa, and bone, based on clinician's knowledge and patient's centered outcomes, such as perception of esthetic conditions, functional limitations, pain/discomfort, root sensitivity, level of sociability post-surgery, and preferences" (Chambrone et al., 2012). Many times there are differences between the esthetic perceptions of dentists and patients.

Contemporary research in parodontology embrase different domains.

Most of current research and innovation involves techniques aimed at *regenerating lost bone and helping the implant surface osseointegrate* once again. Significant progress has been made in recent years to improve the prevention of peri-implantitis, something that is quite positive considering that prevention is the most effective treatment for this condition.

Immunology and microbiology research focuses on the biology, transmission and pathogenesis of viruses and bacteria, as well as on the fundamental immune mechanisms that contribute to both health and disease. There are studies of issues of autoimmunity, emerging pathogens, bacterial and viral pathogenesis, and molecular microbiology, as it relates to auto-immune and infectious diseases of the mouth, including oral cancer and early detection of cancerous and pre-cancerous lesions. Also, large trial studies are concerning inflammation,

cancer biology, virology, diabetes, periodontal disease, dental implants, biomaterials and technologies, advanced imaging, and educational enhancement (Cairo et al., 2011).

As of 1900 there are indications of mucogingival surgical techniques, but more predictable techniques began to appear in the 50s. The first treatments involved a sliding flap operation (Grupe and Warren 1956). According to the displacement direction they can be rotated flaps or coronally advanced flaps. The main limitation of these techniques is the need to have attached gingiva around the area to be treated. They are indicated mainly for the treatment of one tooth/implant. Their main advantages are technical ease and the esthetic results achieved (Zuchelli et al., 2003).

Free grafts were indicated if there was no keratinized tissue. Their main disadvantages are the esthetic results and the management of the palatal area. It is a predictable technique to increase the width of the attached gingiva (Sullivan and Alkins 1968).

In 1974, Karring proved that the characteristics of epithelial tissue are genetically determined by the subjacent connective tissue, which justified the development of connective tissue graft techniques (Karring et al., 1972). They were first described by Edel in 1974, popularized by Langer and Langer in 1985, and modified by several authors (Wennstrom et al., 1996).

They were initially indicated to thicken keratinized gingiva, and are currently indicated for the coverage of gingival recession, the thickening of soft tissue in edentulous areas, the thickening of tissue surrounding implants or teeth, papilla reconstruction, scar correction and modification of periodontal or peri-implant biotype. Connective tissue grafts are considered the gold standard for root coverage given their predictability, stability over time, increase in thickness and length/width of keratinized gingiva (Azzi et al., 2002).

If this technique cannot be applied, a second choice might be coronally advanced flaps combined with allogenic or xenogeneic matrices. The last choice would be coronally advanced flaps or guided tissue regeneration (Roccuzzo et al., 2002).

As there is only limited literature available on plastic periodontal surgery in connection with implants, the results obtained on teeth should be used as a clinical guide for the treatment of peri-implant recession/mucosal defects. Selecting the right type of graft (size and shape) as well as complete root coverage achieved with the coronally advanced flap will enhance the final esthetic results. Connective tissue grafts are an essential tool in periodontal and implant mucogingival surgery, both functionally and esthetically (Reiser et al., 1996).

They are highly esthetic and predictable for root coverage: percentages of complete root coverage reach 89%. There is partial root coverage in 80.94% of cases and complete root coverage in 46.63% of cases. The postoperative process is better with connective tissue grafts than with free graft techniques. The double blood supply to the graft increases its success rate (Pini et al., 2000).

There is a direct correlation between flap tension and reduced root coverage, and between tissue thickness and the percentage of coverage achieved: flaps more than 0.8 mm thick have a better prognosis. Different techniques have been proposed for the use of grafts: tunnel techniques

(Raetzke,1985; Allen, 1994); reposition of the flap partially covering a connective graft with an epithelial border (Langer 1985); coronally advanced flaps with vertical releasing incisions (Nelson 1987; Wennstrom and Zuchelli 1996); or without them (Bruno 1994); or lateral sliding papillae flaps (Harris 2003). In all techniques, graft size is greater than bone dehiscence and the graft is placed and sutured at CEJ level (Bohm et al., 2006).

Connective tissue grafts with epithelial border were used by Langer and Langer. Connective tissue grafts have an exposed section in the techniques described by. The exposed root is usually treated with curettes. In the past, mucoperiosteal flaps were used on the recipient site, but nowadays mucosal grafts are preferred as they allow for greater graft mobility and coverage (Bosco and Días 2007).

Palate harvesting in the area between the canine and the first molar is the procedure of choice. It is there that the palatal mucosa is thickest, as it decreases towards the molar area. It increases from the gingival margin towards the palatal suture. The palatal mucosa is thickest with age and is thinner in women. The thickness of the palatal mucosa and the height of the palatal vault are essential considerations when selecting a graft harvesting technique (Hurzeler and Weng 1999).

The harvesting of an epithelial-connective graft is recommended in the case of thin palates. Once the graft has been harvested, the epithelium is eliminated, the graft is repositioned at the donor site, sutured, and surgical cement is applied. This makes it possible to obtain the graft more superficially, hence avoiding complications in patients with thin biotypes. Replacing the epithelialized graft promotes faster healing. The references to consider are the palatal rugae (anterior area), the palatal root of the first molar (posterior area) and the neurovascular bundle coming from the greater palatine foramen (medially).

Regarding the shape of the palate and the position of the palatine artery, Reiser et al. (1996) identified three possible palatal vaults: shallow, average and high. According to this classification, depending on the size of the arch, the neurovascular bundle is located at 7 mm, 12 mm or 17 mm from the adjacent tooth (Reiser et al., 1996). Hemorrhaging can be avoided by respecting this structure.

The initial critical factor is whether we will obtain a graft with or without epithelial border. At first this border was included to provide a better transition with the existing epithelial border when treating gingival recession. But later it was noted that if the epithelium was maintained, the esthetic outcome was not better, and that the final result depended mainly on the connective tissue graft. Both the natural appearance, shape and color of the new epithelium will depend on the subjacent connective tissue. Harvesting the graft with an epithelial border hinders healing by first intention in the donor site, which leads to pain and potential postoperative bleeding. Acrylic plates and haemostatic drugs have been used to prevent such situations. If the epithelial strip is not harvested with the graft, access can be achieved with one (single incision technique), two (angular-incision technique) or three (trapdoor technique) incisions. If there are more incisions, the connective tissue can be better visualized, but the flap has lower vascularization which may lead to postoperative necrosis. The current trend is to harvest the graft with only one incision (Khoury and Happe 2000).

The single-incision technique has the following advantages: optimal vascularization of the cover flap, a small number of sutures, no need for additional haemostatic or compressive measures, a better postoperative process and the possibility of obtaining grafts of variable dimensions. The palatal sliding flap technique is cited as an alternative to the conventional connective tissue graft. It has a better prognosis because the flap remains vascularized and is easier to stabilize. It is specially indicated when used jointly with bone grafts or membranes that make vascularization harder (Barbosa et al., 2009).

All these different techniques place have in common a connective tissue graft on the root surface to be covered and above it the flap, which provides partial or total coverage. This can be achieved with suturing, but the possibility of using cyanoacrylate has been described with promising results. The same technique would be used on implants with recession.

Post-treatment healing is performed by using connective tissue grafts or epithelial-connective grafts we can achieve the formation of a long junctional epithelium with a fibrous attachment, although a few studies report variable degrees of regeneration. Only the areas where the cementum was preserved were able to form new cementum (Goldstein et al., 2001; Pasquinelli 1995).

Periosteum cannot regenerate after it has been detached from the bone surface, therefore its presence does not seem to condition the type of healing the root surface will have. The mechanical trauma of detaching DE the periosteum from the bone destroys the cell layer called "cambium layer" in the periosteum. This layer has the potential for regeneration, hence the risk run by detaching it (Melcher 1971; Weng et al., 2000).

Selection of the appropriate procedure, and precise, meticulous surgical technique, will provide successful and highly predictable results in the treatment of gingival recessions. For these is needed to continue the research in the field of prevention, early diagnosis and appropriate treatment for any of the different types of periodontal disease.

The four published articles that were included in establishing this research direction have the following cumulative impact factor (CIF):

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

1. Martu, M.-A.; **Luchian, I.;** Mares, M.; Solomon, S.; Ciurcanu, O.; Danila, V.; Rezus, E.; Foia, L. The Effectiveness of Laser Applications and Photodynamic Therapy on Relevant Periodontal Pathogens (*Aggregatibacter actinomycetemcomitans*) Associated with Immunomodulating Anti-rheumatic Drugs. *Bioengineering* **2023**, *10*, 61. https://doi.org/10.3390/bioengineering10010061
2. **Luchian, I.**; Budală, D.G.; Baciu, E.-R.; Ursu, R.G.; Diaconu-Popa, D.; Butnaru, O.; Tatarciuc, M. The Involvement of Photobiology in Contemporary Dentistry—A Narrative Review. *Int. J. Mol. Sci.* **2023**, *24*, 3985. https://doi.org/10.3390/ijms24043985
3. Sufaru, I.-G.; Martu, M.-A.; **Luchian, I.;** Stoleriu, S.; Diaconu-Popa, D.; Martu, C.; Teslaru, S.; Pasarin, L.; Solomon, S.M. The Effects of 810 nm Diode Laser and Indocyanine Green on Periodontal Parameters and HbA1c in Patients with Periodontitis and Type II Diabetes Mellitus: A Randomized Controlled Study. *Diagnostics* **2022**, *12*, 1614. https://doi.org/10.3390/diagnostics12071614
4. Goriuc, A.; Foia, L.; Cojocaru, K.; Diaconu-Popa, D.; Sandu, D.; **Luchian, I.** The Role and Involvement of Stem Cells in Periodontology. *Biomedicines* **2023**, *11*, 387. https://doi.org/10.3390/biomedicines11020387

1.9. From old to new concepts of photobiology in contemporary dentistry

1.9.1. Brief history on light theories

While modern physics has succeeded in breaking down the components of nature into ever tinier and more exotic parts, light itself remains unchanged. Healing with light, often known as heliotherapy or phototherapy, has been practiced for thousands of years (Kern and Lewy 1990).

Before the development of antibiotic medicines, increased sun exposure in sanatoria was a common therapy for infectious respiratory disorders such as tuberculosis (TB) (Sabra 1981). However, one question remains: what is light, exactly? This brings us to a single aspect of light's miraculous nature: it has no volume. Additionally, unlike negatively charged electrons, photons do not repel one another when packed into a tiny area. So, how many light angels can dance on the head of a pin? In theory, there is no limit.

Edgar Cayce and Rudolph Steiner were only two of the visionaries from the last century who foresaw the medical revolution that vibrational healing from color and light would bring (Sabra 1981). Ever since, growth in the sector has been meteoric, and now light is employed in all kinds of areas, from laser eye surgery to telecommunications.

As difficult as it is to comprehend light, it was far more challenging for the ancients. "Light is the activity of what is transparent," Aristotle stated, quite cryptically (Subbarayappa 2001). This transparency was a vital quality of many substances; when triggered by the sun or fire, it generated light and color. Empedocles, a fifth-century B.C. philosopher and poet, offered the great insight that light is a flowing material released by the sun and that we are unaware of its

movement because it travels too quickly. Ancient Greek mathematicians, including Plato and Euclid, believed that the eyes emit some form of visual ray (Murphy et al., 2002).

Alhazen's idea of the camera obscura (in Latin "dark room") used a small opening to project an inverted image of the outside world onto a wall. Leonardo da Vinci made the connection between the eye and the camera, a device invented centuries later. Later, Descartes performed a somewhat spectacular inspection of an ox's eyeball, scraping away the back of the eye and gazing into it. He noticed that the eye captures an inverted, upside-down representation of the world (Metwaly et al., 2021; Rueggeberg et al., 2017).

Light immediately flowed into Isaac Newton's laboratory and was never the same again. Then, in the 1660s, Newton established that white light is composed of all the hues of the spectrum. He used a prism to break sunlight into a rainbow, then used another prism to cohere the colors back into white light. One of the most important light-related discoveries was made by a Scot named James Clerk Maxwell in the 1860s. Maxwell had been investigating electricity and magnetism and discovered that they traveled through space at the speed of light. Light, he concluded, is an "electromagnetic" wave (Cocilovo 1999; Liebert and Kiat 2021).

Clearly, light will continue to be highly useful in business, research, art, and our everyday, ordinary movements. Light pervades our experience at all levels of life. It is a wonderful instrument: a bearer of beauty and a source of life.

- **Spectrum of Light: From Visible to Invisible**

Scientists create physical process models to better explain and predict behavior. The same is true for light energy. Photons simulate the particle-like characteristics of light. A photon has neither mass or charge. It transports electromagnetic energy and interacts with other discrete particles, such as electrons, atoms, and molecules (Qureshi et al., 2017). Light (or similar radiations) is constituted of numerous 3D matter corpuscles moved by associated (electromagnetic wave-like) distortions in a universal medium.

Therefore, light exhibits all the characteristic properties of photons:

• The number of photons per unit time indicates the amplitude of light;

• The frequency of photons in a ray of light shows its intensity and color;

• The direction of spin of photons in a ray of light indicates its polarity.

A light source is represented by a continuous flow of photons, each of which has a certain energy that varies with its wavelength. The colors blue and red are only two examples of the spectrum of light's wavelengths. Light waves are complex and carry light energy with them (Rkein and Ozog 2014). Light's intensity, propagation direction, frequency or wavelength spectrum, and polarization are its most defining features.

When light travels through a medium, it interacts with that medium. The most important interactions are absorption and scattering. Absorption is a transfer of energy from the electromagnetic wave to the atoms or molecules of the medium, while scattering is the redirection of light caused by the light's interaction with matter (Voit et al., 2009; Hohmann et al., 2014; Jacques 2013).

Light, or electromagnetic radiation (EMR), may be thought of as a continuous wave of light particles. These photons, or "light particles," oscillate and spin as they travel through space (Nothelfer et al., 2018).

Generally, electromagnetic radiation (EMR) is classified by wavelength into radio waves, microwaves, infrared, the visible spectrum that we perceive as light, ultraviolet, X-rays, and gamma rays. Visible light is usually defined as having wavelengths in the range of 400–700 nanometers (nm) between the infrared (longer wavelengths) and the ultraviolet (with shorter wavelengths), known as UV, as seen in Figure I.3.1. (Sliney et al., 1976; Wiest et al., 2015).

Figure I.3.1. Light spectrum.

As a result of differences in vibration frequency and photodynamic reactivity, the effects of photons in various parts of the electromagnetic spectrum (EMS) on organic and inorganic materials are also quite different. Radiation with shorter wavelengths, such as gamma rays and X-rays, has a tendency to ionize materials; radiation with longer wavelengths, such as radio waves, is comparatively harmless (Hohmann et al., 2014; Jacques 2013; Nothelfer et al., 2018).

- **Light in Modern Dentistry**

Infrared Light and Near Infrared Light

While visible and ultraviolet light may only penetrate the skin and deeper layers of tissue by a few microns apiece, infrared radiation can travel 20–30 mm through a variety of tissues and may have far more profound effects, causing changes in circulating cytokines (Yeh et al., 2019).

Dental tissue optics has been created as a noninvasive tool for early caries diagnosis as part of the ongoing quest for more precise diagnostic procedures. Novel and promising optical imaging uses near-infrared (NIR) light for the early detection of dental caries lesions and the evaluation of lesion severity (Fried et al., 2005). The technique is noninvasive, does not include the use of ionizing radiation, and is thought to be more sensitive to early demineralization than dental radiography.

The NIR (1310 nm) imagery was compared to radiography images by Jones et al. (2003). Poor radiographic contrast was seen for simulated approximal lesions in tooth pieces of various thicknesses. When the lesion was irradiated with near-infrared light, however, the line between the lesion and the healthy enamel around it stood out sharply.

Bühler et al. conducted an analysis in which imaging reflecting occlusal caries lesions was compared with radiographic imaging to evaluate the potential of an NIR laser in the identification

of early occlusal caries lesions. NIR pictures were again shown to be superior to radiographic images, as was the case in the aforementioned investigation (Buhler et al., 2005).

Compared to radiography, all of these investigations showed that the NIR TI approach was highly sensitive and specific. The method has the potential to enhance the regular monitoring of enamel lesions during preventative intervention, and it can be valuable for early caries diagnosis and patient follow-up (Abogazalah and Ando 2017).

High-Energy Visible (HEV) Light

High-energy visible (HEV) light, also known as "blue light," is the term used to define light with a wavelength between 400 and 500 nm and lower energy than UV (Yin et al., 2013). Due to blue light's superior safety profile compared to UV light and its comparatively reduced photodegradation of the molecules it irradiates, it has attracted a great deal of interest as a potential therapy for a variety of conditions (Cabral 2019).

In vitro and in vivo studies have shown that blue light is deadly to bacteria and can kill both Gram-negative and Gram-positive microorganisms. The 402–420 nm spectral range is the most effective antibacterial spectral range; however, it has also been observed that the 455 nm and 470 nm wavelengths have antimicrobial potential against some bacterial species (e.g., S. aureus) (dai et al., 2012). Additionally, it has been determined that blue light can kill the anaerobic oral pathogens Prevotella, Porphyromonas, and Fusobacterium (Feuerstein et al., 2005).

The absorption of electromagnetic energy in the blue light spectrum by electrons in molecular orbitals can cause photochemical processes or the internal conversion of light to heat. Since this is the case, it is plausible that the employment of light irradiation units in dental therapy warms the oral tissues. It is common knowledge that the mucous membranes of the lips are particularly vulnerable to the high temperatures produced at the irradiators' tips (Spranley et al., 2012). A rubber dam will not prevent mucosal injury from occurring due to direct heat stimulation. As a result, during dental restoration treatments, the only place the light points should be placed is directly over the restoration. However, a common curing lighting with an output power of >600 mW/cm2 is currently utilized in clinical practice.

As a safety measure, dentists should be aware that irradiation time during blue-light- based operations should be kept to a minimum. It is also important to monitor how far away the irradiation source is from the target. Some dentists believe that alternative strategies, such as keeping some space between the light's tip and the tooth in question or dialing back the irradiance, can help to prevent heat injury to the pulp. Nevertheless, the curing of composite resin in low-light portions of the oral cavity, such as in the cervical regions of class II cavity preparations, might be compromised if the light tip's radiant exitance values decrease as distance from the target tooth increases (Jandt and Mills 2013; Price et al., 2014).

There has been an increase in the demand for tooth whitening as patients place greater emphasis on the aesthetics of their mouth and teeth, including the color of their teeth. One of the most common whitening techniques involves applying a solution containing 25–40% hydrogen peroxide to the teeth's surfaces. Halogen curing lights, LEDs, diode lasers, argon lasers, and plasma arc lamps are just some of the blue-light-producing units used in tooth bleaching, which

have been developed to improve the activation of hydrogen peroxide in a shorter amount of time and thus produce more desirable cosmetic results (Yoshino and Yoshida 2018; Fluent et al., 2019; Oliveira et al., 2022). For this reason, blue light is crucial in contemporary dental care.

Ultraviolet Light

Extreme ultraviolet (below 100 nm), far ultraviolet (100–200 nm), middle ultraviolet (200–300 nm), and near ultraviolet (300–380 nm) are the four zones often used by physicists to investigate UV photons. However, three groups are commonly identified based on UV interactions with biological materials. The wavelengths of ultraviolet (UV) C light are between 100 and 280 nanometers, those of ultraviolet (UV) B light are be- tween 280 and 315 nanometers, and those of ultraviolet (UV) A light are between 315 and 380 nanometers (Maclean et al., 2016).

Dermal 7-dehydrocholesterol absorbs ultraviolet light between 290 and 315 nm, chang- ing it into pre-vitamin D3, which then isomerizes into vitamin D3 and aids the immune system (Grober et al., 2013).

Ultraviolet radiation (405 nm) has several uses beyond just stimulating vitamin D production; it may also be used to disinfect or sterilize surfaces, air, and water, eradicating Pseudomonas aeruginosa in plumbing systems. Germs are killed by ultraviolet (UV) radiation because it damages DNA, forming thymine dimers that are difficult to repair (Gerba 2015).

Microorganisms are killed by ultraviolet light at far lower irradiation fluences than those caused by visible light. UV-C wavelengths about 260 nm are particularly effective for this purpose. UV light's high photon energy means that it does not take much irradiance to create detrimental consequences, despite claims that the advantages of UV at low doses vastly exceed its adverse impact (Dai et al., 2011; Wang et al., 2021).

The oral cavity provides a favorable setting for the clinical use of ultraviolet UV irradiation technology for the management of polymicrobial biofilms in periodontal and peri-implant microbiomes (Conner-Ker et al., 1998; Thai et al., 2005). However, endodontic infections and inflammation in root canals may be where it clearly emerges (De Brito et al., 2020; Delikan et al., 2021). Infected root canals could potentially be treated with UV irradiation (Metzger et al., 2007; Morio et al., 2019; Stajer et al., 2020), and we believe that the application of narrow UV spectra will destroy microorganisms, stimulate tissues and cells, and cause the release of chemokines, cytokines, and biomarkers (CCBMs) that maintain periapical tissue health.

In 2019, Morio et al. analyzed the cytotoxic effects of 255 and 405 nm UV LED on human embryonic palatal mesenchyme (HEPM) cells and gingival fibroblasts in addition to evaluating the antimicrobial killing effects of 255 and 405 nm UV LED on E. faecalis. E. faecalis was considerably less likely to survive after being treated with 255 nm LED than 405 nm LED.

The trade-off between UV irradiation's antibacterial effectiveness and its safe usage and detrimental effects on host tissues has long been a source of worry in both medical and dental contexts. Reed eloquently pointed out that UV irradiation's potential to generate tissue and cell damage is strongly linked to the depth to which it can penetrate (which is determined by the wavelength) (Metzher et al., 2007).

In contrast with UV-A and UV-B, UV-C is the most biologically active radiation, and it poses no risk to people. In contrast with the deeper penetration of UV-B and UV-A rays, UV-C

rays are absorbed by the human skin's outermost dead layer. The practical designs of ultraviolet germicidal irradiation fixtures are also improving, becoming more efficient while remaining safe, but new ideas are required to further increase efficiency and effectiveness while keeping production expenses low (Reed 2010).

Researchers have been able to reevaluate the antimicrobial and cytotoxic properties of very specific wavelengths and spectra thanks to the availability of new UV LED, allow- ing them to pinpoint the wavelengths, powers, and doses of irradiation that maximize antimicrobial activity while minimizing cytotoxicity to tissue and cells. Reports reveal that optimum antibacterial activity takes place between 255 and 280 nm, whereas general antimicrobial activity occurs between 200 and 400 nm (Bruls et al., 1984; Schafer et al., 2008; Yamano et al., 2020).

For instance, during the pandemic period, the American Dental Association (ADA), as well as most European dental organizations, recommended the use of UV lamps and other air purifiers and high-efficiency aspiration during treatments (Giraudeau 2021; Devlin et al., 2021).

Shortest Wavelengths-X-ray

The wavelength of an X-ray is just 0.01–10 nm, much shorter than the wavelength of UV light. In the medical field, X-rays are primarily associated with imaging proce- dures including traditional X-rays, CBCT, and OPG. DNA damage and tissue destruction, which can be either pathological or therapeutic, are more likely to occur with shorter light beams (Gianfaldoni et al., 2017). X-rays have obviously made the transition from the world of unseen enigmatic energy to everyday conventional medical practice; depending on how they are employed, they can inflict significant damage or deliver great advantages (Cogneta et al., 2012).

Light-Sources in Dentistry Lasers. LASER is the abbreviation for Light Amplification via the Stimulated Emission of Radiation. Since Miaman first introduced the use of the laser in dentistry in the 1960s (Gross and Hermann 2007), researchers have been exploring its many potential clinical uses. Soft or cold lasers, based on semiconductor diode devices, are compact, low-cost devices used predominantly in ap- plications, while hard lasers, such as carbon dioxide (CO2), neodymium yttrium aluminum garnet (Nd:YAG), and erbium doped yttrium aluminum garnet (Er: YAG), offer both hard tissue and soft tissue applications, but have limitations due to their high costs and their potential to induce thermal damage (Walsh 2003).

Mechanism of Action

Dental lasers transmit light to the tissue through an active medium, which can be a gas, a crystal, or a solid-state semiconductor. This is the primary factor in determining the laser's wavelength and other features.

Laser-generated light energy can interact with tissue in four distinct ways (Theodoro et al., 2021; Kikuchi et al., 2022). All four of these processes: reflection, transmission, scattering, and absorption, are possible.

- **Types of Lasers**

Carbon Dioxide Laser (CO2)

Since the CO2 laser wavelength is highly selective for water, it may quickly and effectively remove soft tissue and stop bleeding with minimal penetration (Fujiyama et al., 2008). Soft tissue

surgery is best performed using CO2 (10,600 nm), Nd:YAG (1064 nm), DL (800–980 nm), Er:YAG (2940 nm), or Er,Cr:YSGG (2780 nm) lasers. In addition to being a very conservative technique, its benefits include an increase in tissue temperature that aids in hemostasis and decreases microbial growth (Saglam et al., 2017).

Neodymium Yttrium Aluminum Garnet Laser (Nd:YAG)

This is a highly efficient surgical laser because the Nd:YAG wavelength is well ab- sorbed by pigmented tissue. Research on the use of the Nd:YAG laser for nonsurgical sulcular debridement in periodontal disease management has been conducted in addition to its surgical applications (Aoki et al., 2008).

Erbium Lasers

Lasers of the erbium "family" come in two varieties, one with a longer wavelength (Er, Cr: YSGG; yttrium scandium gallium garnet) and the other with a shorter one (Er: YAG; yttrium aluminum garnet). Erbium lasers have the largest absorption of water and the strongest affinity for hydroxyapatite of any dental lasers. Because of this, they are the preferred laser for restoring dental hard tissues (Oliveira et al., 2012).

In vitro investigations have demonstrated that both Er:YAG and Er,Cr;YSGG lasers cause alterations in the surface morphology of treated roots, making them more irregular and rough (Theodoro et al., 2002).

Diode Lasers

Diode lasers generate laser wavelengths in the 810 nm to 980 nm range using a solid-state semiconductor active medium composed of aluminum, gallium, arsenide, and sometimes indium.

Aesthetic gingival re-contouring, soft tissue crown lengthening, soft tissue impacted tooth exposure, inflamed/hypertrophic tissue removal, frenectomies, and photostimu- lation of aphthous/herpetic lesions are only a few of the operations that fall under this category (Lesniewski et al., 2022).

For noninvasive periodontal therapy, dentists can employ a combination of diode lasers (DL) (808–904 nm) and neodymium-doped yttrium–aluminum–garnet (Nd:YAG; 1064 nm), erbium-doped yttrium–aluminum–garnet (Er:YAG; 2940 nm), and erbium- chromium (Pawelczyk-Madalinska et al., 2021).

Sulcular debridement (the removal of the sulcular epithelium from the periodontal pocket) and the promotion of the decrease in supra- or subgingival periodonto pathogenic bacteria are both indications for the use of the Nd:YAG laser and the DL, respectively (Slot et al., 2009).

The use of lasers in hard tissue applications and soft tissue surgeries has progressed to a highly refined stage over the course of several decades, and additional refinement is possible. Laser-based photochemical reactions' ability to zero in on individual cells, pathogens, or chemicals has also been highlighted for potential use in the future.

Taking into account the classification of Placek and according to recent literature, it seems that the diode laser surgery techniques are superior in terms of haemostasis, surgical time, pain, edema, post-surgical inflammation, and healing time when compared with conventional surgery (Inchingolo et al., 2023).

1.9.2. Dental Therapeutic Strategies

- Photodynamic Therapy

Unlike other methods of tissue and cell death, photodynamic treatment (PDT) is both noninvasive and extremely specific. It relies on the fact that a photosensitizer (PS), molecular oxygen, and visible or near-infrared (NIR) light must all be present simultaneously, although none of these things are lethal or harmful to cells or tissues on their own. In an ideal scenario, the PS is absorbed and stored mostly in the intended cells (Sperandio et al., 2013; Kwiatkowski et al., 2018).

Mechanism of the Photodynamic Action

The photodynamic response can occur via two major processes. Both rely heavily on oxygen molecules within living cells. Both methods have the same initial phase. After entering the cell, a photosensitizer absorbs photons at a certain wavelength that corresponds to its absorption spectrum (AS), causing it to transition from its ground (singlet) to its excited (singlet) energy state (S◦ to S1). While some of the energy is lost as fluorescence, the rest is used to excite a photosensitizer molecule into its active triplet state, T1. (Figure I.3.2.).

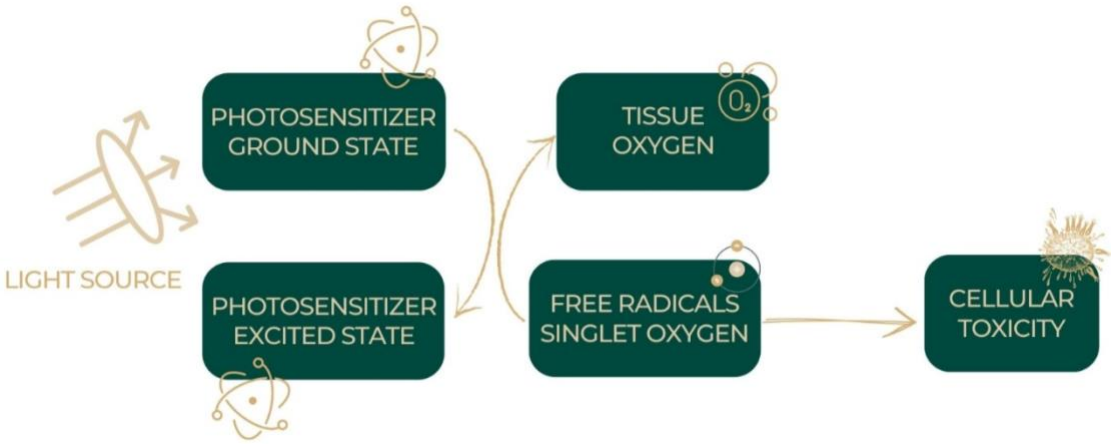


Figure I.3.2. PDT mechanism.

- PDT in Dentistry

Xu et al. (2009) found that a 5 min exposure to 665 nm laser light at 20 and 40 mW/cm2 was sufficient to kill endodontic bacteria in vitro. Gingival fibroblasts and osteoblasts showed no apoptotic changes after laser therapy, demonstrating the safety and efficacy of PDT.

PDT (665 nm, 60 J/cm2) applied in vitro reduced the lifespan of E. faecalis in dental root canals by 77.5% (Foschi et al., 2007). A five-log decrease in microbial growth was seen in recent research, which used a combination treatment of root canal surgery and PDT (660 nm, 15 J). In contrast with conventional endodontic surgery, this approach yielded better results. Furthermore, after 36 months of therapy, the periapical infection area decreased by 78% (Garcez et al., 2015).

Numerous studies have demonstrated that PDT is an effective supplement to scaling and root planning for the treatment of aggressive periodontitis (Vohra et al., 2016). Adolescents are more prone to developing a localized type of aggressive periodontitis, a rare illness. The

subgingival microbiota of young people with periodontitis contains a greater concentration of actinomycetemcomitans according to research by Zambon and colleagues (Zambon et al., 1983).

Park et al. (2019) demonstrated the feasibility of toluidine blue O (TBO)-mediated PDT as a noninvasive supplementary method for the treatment of periodontitis. Many bacteria, including certain oral microbes, can be inactivated by PDT, according to some in vitro investigations (Salvi et al., 2020; Lopez et al 2020; Rossi et al., 2022; Munteanu et al., 2022). Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia, and Streptococcus sanguis, all of which have been linked to periodontal disease, were demonstrated to be strongly inhibited by aPDT under a variety of settings (Sgolastra et al., 2013).

Park et al. (2020) found that PDT was selective against periodontitis pathogenic bacterial while having no effect on resident oral bacterial growth. Moreover, its cytotoxic impact on normal periodontal cells was shown to be below the level typically seen in antiseptics.

For localized infections, the therapeutic use of PDT is thought to cause minimal harm, as confirmed by Hu et al. (2018). These results are consistent with PDT being the mechanism responsible for killing off microorganisms. When a certain wavelength of laser interacts with a photosensitizer dye, the dye molecule is excited and oxidized to generate singlet oxygen, leading to a reduction in the total number of bacteria (Huang et al., 2019).

Antifungals, both topical and oral, are commonly prescribed to treat stomatitis. How- ever, this disease is common because of antifungal drug resistance, and it tends to reoccur. Prolonged and frequent usage increases the risk of strain resistance to pharmacological treatments and unwanted consequences (Whaley et al., 2016; Senna et al., 2018). Denture stomatitis can be a painful condition; however, antimicrobial photodynamic treatment (PDT) has been identified as a potential solution (Alrabian et al., 2019).

The use of antimicrobial PDT is desirable because it avoids the need for standard medication therapy, shortens the duration of treatment, and prevents the need for extended drug intake or excessive drug dose (Javed et al., 2014; Etcheverry et al., 2016). Using phototherapy in conjunction with nystatin was designed to drastically lower colony numbers, as opposed to only using the antifungal agent. Because of the alterations induced by PDT, nystatin is better able to enter fungal cells and bind to the ergosterol in their membranes, resulting in cell destruction and necrosis (Afroozi et al., 2019).

Always on the lookout for new ways to improve the accuracy of chemo-mechanical root canal cleaning, endodontists have developed several adjunctive techniques. As a result, PDT methods were offered as an alternative method to standard root canal endodontic preparation. This is carried out with the goal of eliminating as many germs as possible from the root canal structures (Plotino et al., 2019; Vendramini et al., 2020). Many variables have been linked to its success.

Considering this, photodynamic treatment is gaining favor not just with doctors, but with patients as well. The rapid progress and advancements in technology and optoelec- tronic equipment might promote the use of the photodynamic approach in clinical care. Although antibiotics are often used to treat periodontitis and peri-implantitis, PDT appears to be a safer alternative.

- Photobiomodulation (PBM)

Photobiomodulation, also known as low-level light therapy (LLLT), has been attracting attention in the dental field due to its potential to improve a wide range of oral conditions.

Due to its anti-inflammatory, antinociceptive, and antibacterial properties, photobiomodulation therapy (PBMT) has emerged as a potential and successful therapeutic option for the management of oral mucositis, periodontology, temporomandibular disorder (TMD) pain, dental implant osseointegration, and orthodontic tooth movement, which has been investigated in several studies (Anders et al., 2015, Mester 2017, Nejat et al., 2021).

In the systematic review, Zadik et al. (2019), studied the effectiveness of LLLT in the management of oral mucositis and associated pain in cancer patients, which is a frequent side effect of chemotherapy and radiation treatment. According to the study, using specific LLLT settings can be recommended for the prevention of oral mucositis.

Injured nerves produce inflammatory mediators of the arachidonic acid family; how- ever, low-level laser treatment has been found to decrease this production while also stimulating neuronal maturation and regeneration (Badeaux et al., 2015; Modrak et al., 2020).

Theodoro et al. examined the use of LLLT in the treatment of periodontal disease. LLLT combined with non-surgical periodontal therapy was found to be helpful in lowering inflammation, accelerating bone and gingival tissue regeneration, and reducing periodontal surgery postoperative discomfort. Several studies have shown that PBM therapy effectively reduced pain and improved outcomes in patients with temporomandibular disfunction (TMD) and dental implant osseointegration (Dadjoo et al., 2022). According to Zheng et al. (2021), LLLT therapy can effectively accelerate orthodontic tooth movement and minimize the discomfort associated with traditional orthodontic treatment. A recent systematic review has indicated that utilizing LLLT can be an effective method to decrease tooth sensitivity after dental bleaching (Carneiro et al., 2022).

- Photodynamic Inactivation

One of the most well-known light-based treatments used to kill bacteria and other microbes is called photodynamic inactivation.

Microorganisms are susceptible to photo-inactivation if three conditions are met: oxygen, a photosensitizer that can convert light energy into some harmful downstream product(s), and light of the proper wavelength matched with the absorption spectrum of the photosensitizer.

Every photosensitizing dye used in PDI, whether methylene blue, Rose Bengal, hy- pericin, xanthenes, etc., has a slightly different molecular conformation, and each is paired with a certain wavelength that generates a significant enough amount of reactive oxygen species (ROS) to kill the target microorganism (Costa et al., 2008).

As early as the 1920s, Schultz and Krueger used light waves and methylene blue to kill Staphylococcus bacteria, demonstrating the principle of photodynamic inactivation of microorganisms (Schultz 1928).

Despite these early accomplishments, therapeutic interest in photodynamic inactivation of bacteria and viruses has recently emerged in the last 50 years, as research in the subject has been

substantially advanced by technological discoveries that have given rise to more efficient light sources.

1.9.3. Applicability of photodynamic therapy in periodontitis

1.9.3.1 *Introduction*

There are numerous studies in the literature showing promising results for photodynamic disinfection therapy (PDT) with various light sources ranging from blue to infrared against the main pathogens of periodontal disease (Akram et al., 2017, Pummer et al., 2017, Mocanu et al., 2021). Periodontal disease is a chronic inflammation of the deep periodontal tissues caused by specific bacteria that adhere and grow on the surfaces of the teeth and that can also generate pathologies of the dental pulp, and, thus, is involved in numerous systemic diseases, including pneumonia, cardiovascular diseases, renal disease, Alzheimer, autoimmune diseases, cancer, etc. (Kapila et al., 2021). Considering the similarities between aspects of the pathogenesis of periodontal disease and certain autoimmune diseases, such as rheumatoid arthritis, it is of interest that researchers in the field of periodontology are aware of the therapeutic options available (González-Febles and Sanz 2021). Currently, treatment of periodontitis consists of classic non-surgical and surgical treatments, and also various types of non-conventional therapies, such as physical stimulation (thermal treatment or ultrasound), the application of chemical and antibiotic agents and photostimulation (laser/LED treatment).

Regarding phototherapy, it has been reported to accelerate wound healing and reduce post-operative pain and discomfort when using low-level laser radiation (LLLT) (Zhao et al., 2021). *Aggregatibacter actinomycetemcomitans* IgA and IgE levels are significantly higher in the serum and saliva of periodontitis patients (Isola et al., 2020). Moreover, a study observed an increased in vitro resistance of *A. actinomycetemcomitans* to amoxicillin, azithromycin and metronidazole, some of the most frequently prescribed antibiotics in periodontitis patients (Ardila et al., 2022).

Considering the current context of the evolution of bacterial species' resistance to antibiotics and other antimicrobial agents such as triclosan and chlorhexidine, a major objective is to develop other antimicrobial approaches able to inactivate pathogens with considerable effectiveness. A possible option for the local eradication of bacteria is their photodynamic inactivation. By this method, the bacteria are incubated with a photosensitizer and are subsequently irradiated with light of a suitable wavelength. Exposure of the photosensitizer to the light treatment leads to the generation of reactive oxygen species that kill the bacteria through a so-called "oxidative explosion" phenomenon.

Alternatively, irradiation with a diode-type laser is a similar method, but it acts through other biochemical and biophysical mechanisms and does not use a photosensitizing agent, but instead produces the intended therapeutic effect through the nature of the interactions established between the light radiation and the periodontopathogenic bacteria as well as the host tissue (Sculean et al., 2021). Low-level laser radiation therapy (LLLT) supports the production of an increased amount of adenosine triphosphate at the mitochondrial level, thus facilitating the proliferation of fibroblasts, the release of growth factors and the synthesis of collagen (Colaco et al., 2018; Rola et al., 2022).

At the same time, in vitro and animal studies have revealed that this therapy suppresses inflammation in the periodontal tissue by modulating the local immune response and reducing the production and release of certain pro-inflammatory cytokines, such as the tumor necrosis alpha factor (TNF-α), interleukin-1β (IL-1β) and prostaglandin E2. In addition, it has been found to improve local microcirculation through angiogenesis and vasodilation, thus alleviating tissue edema and inflammation (Ren et al., 2017). Although there are recent studies in the literature analyzing the effect of laser therapy and PDT in periodontal disease, the eradication mechanisms of the involved bacteria are insufficiently explored (Table I.3.1.)

Table I.3.1. Studies that analyze the effect of laser and photodisinfection therapy on periopathogenic bacteria in vitro.

Technique	Bacteria	Effect	Author
PDT + toluidine or methylene blue dye	*A. actinomycetemcomitans*	100% eradication at 10 mg/mL	Valle et al., 2019 [14]
PDT + rose bengal	*P. gingivalis, A. actinomycetemcomitans, F. nucleatum*	Maximal reduction at 160 μg/mL rose bengal	Wang et al., 2021 [15]
Diode Laser 810-nm	*A. actinomycetemcomitans*	93% reduction at 2.5 W; 30 s	Tantivitayakul et al., 2018 [16]
Diode laser 635 nm + phycocyanin	*P. gingivalis*	Mean reduction 44.24%	Etemadi et al., 2022 [17]

Moreover, no study to date has analyzed the effect of diode laser therapy and that of photodynamic treatment on a periodontopathogen in the context of anti-TNF-α immunomodulatory therapy. Considering the immunocompromised status of some patients undergoing immunosuppressive therapy and the potential drug interactions that can be established between the antimicrobial agents applied at a systemic level, the research of local, minimally invasive methods of inactivating periodontal pathogens in the context of these systemic therapies with modifying drugs of the immune response such as anti-TNF-α is justified.

Considering the multitude of therapies used in the treatment of autoimmune diseases such as rheumatoid arthritis (RA), ankylosing spondylitis, psoriasis, Crohn's disease and others, that have the role of modulating the host's response, the aim of the current study is to investigate to what extent they may influence the inactivation capacity of certain adjuvant therapies used in periodontal treatment on a critical periodontopathogen such as *Aggregatibacter actinomycetemcomitans*.

1.9.3.2 *Material and methods*

This in vitro study evaluated the antimicrobial effectiveness of the laser radiation emitted by a diode-type laser device (Epic X, Biolase, Foothill Ranch, CA, USA), wavelength 940 nm, continuous mode, 300 μm insert, 9 mm, non-initialized, and also a photodisinfection device (Helbo® Photodynamic Systems GmbH & Co KG, Senden, Germany), 670 nm, 75 mW/cm^2, light spot 0.06 cm in diameter. For the photodisinfection therapy, we used HELBO Blue photosensitizer®, a liquid containing methylene blue (3,7 dimethyl phenothiazine chloride), which has an absorbance maximum at 670 nm. These minimally invasive therapies were applied

to a type strain of *Aggregatibacter actinomycetemcomitans* (DSM-8324) in the presence or absence of active substances used in the therapy of rheumatic pathology.

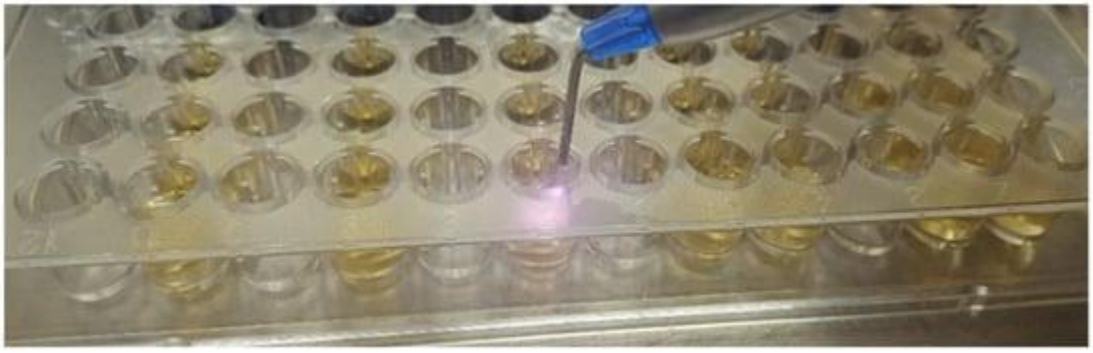
For this purpose, microplates with 96 wells (Deltalab, Rubi Barcelona, Spain) were used, in which 100 μL of the dilutions of the active substances prepared in advance were pipetted, using as a vehicle a brain-heart infusion broth (Bio-Rad, Hercules, CA, USA) supplemented with 20% human blood serum inactivated at 56 °C for 30 min.

The following active substances were tested, in concentrations equivalent to those reached in the plasma of subjects treated with the following dosage regimen:

- Etanercept 2.5 μg/mL (E)
- Infliximab 50 μg/mL (I)
- Metotrexat 2.5 μg/mL (M)
- Etanercept 2.5 μg/mL + Metotrexat 2.5 μg/mL (E + M)
- Infliximab 50 μg/mL + Metotrexat 2.5 μg/mL (I + M)

Next, 100 μL of bacterial inoculum (10^5 CFU/mL) prepared extemporaneously in the same type of liquid medium, using a 24 h culture on Tryptone Soya Agar (Bio-Rad, Hercules, CA, USA) were pipetted into each well.

In parallel, control wells were also prepared (positive-CP, respectively negative-CN) to evaluate the bacterial growth respective to the sterility of the growth medium. Some of the wells were treated with a Diode type laser (two power regimes: 1 W and 5 W respectively) and with



photodisinfection (Figure I.3.3. and I.3.4.). Each well was treated for 30 s.

Figure I.3.3. Application of diode laser treatment to *A. actinomycetemcommitans*.

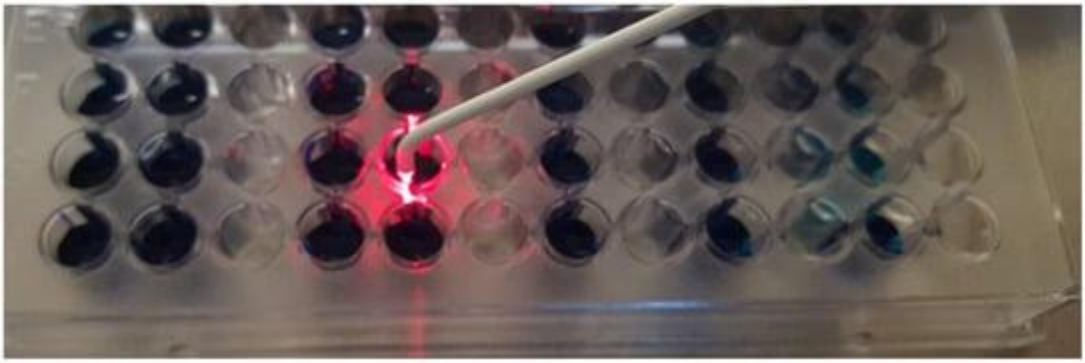


Figure I.3.4. Application of photodisinfection treatment to *A. actinomycetemcommitans*.

The microplates were then immediately placed in the incubator at 36 °C, ensuring microaerophilic conditions with the help of GENbox anaer sachets (bioMerieux, Craponne, France). After 48 h of incubation, in order to evaluate the degree of bacterial growth, the absorbance of each well was read at a wavelength of 492 nm, with the help of an MR-96 spectrophotometer (Mindray, Shenzhen, China), with the exception of the wells pretreated with photoactivator, in which the interpretation was made by seeding a volume of 10 µL on solid medium and re-incubating (in the case of the positive control, serial dilutions were practiced for the exact determination of the bacterial load existing at the time of the laser treatment in the wells containing photoactivator).

The reduction in turbidity in the wells seeded with the bacterial inoculum and implicitly of the absorbance of their content, correlates with the inhibition of microbial growth. The interpretation was made by comparison with the absorbance of the positive control wells, considered the standard of microbial growth (100%). In the case of wells containing photoactivator, the degree of reduction was calculated by referring to the number of CFU (colony-forming units) obtained from the cultivation of the positive control.

The data obtained in relation to CFU/mL were logarithmically transformed. In order to identify the differences between the groups within the transformed data, the Kruskal–Wallis one-directional nonparametric analysis of variance was performed. A U–Mann–Whitney test was applied to compare the differences between any two groups. Statistical comparisons were performed using SPSS software version 19 (SPSS, Chicago, IL, USA). For all analyses, $p < 0.05$ was considered statistically significant.

1.9.3.3 *Results*

In the case of wells not treated with laser or photodisinfection, the degree of growth reduction was insignificant, the variations being less than 10% compared to the positive control in the case of all active substances and their combinations (Table I.3.2.)

In the case of laser application, the reduction in microbial multiplication was evident, with variations depending on the treatment method (Table I.3.3.).

Table I.3.2. Values of *A. actinomycetemcomitans* for the wells not submitted to adjuvant therapy.

Active Substance	E	E + M	I	I + M	M	CP	CN
Absorbance value (mean and standard deviation)	2.054 ± 0.197	1.922 ± 0.189	2.031 ± 0.214	1.968 ± 0.191	2.075 ± 0.174	2.163 ± 0.209	0.154 ± 0.028

Etanercept 2.5 µg/mL (E); Infliximab 50 µg/mL (I); Metotrexat 2.5 µg/mL (M); Etanercept 2.5 µg/mL + Metotrexat 2.5 µg/mL (E + M); Infliximab 50 µg/mL + Metotrexat 2.5 µg/mL (I + M).

Table I.3.3. Values of *A. actinomycetemcomitans* for the wells submitted to adjuvant laser therapy and photodynamic therapy.

Active Substance	E	E + M	I	I + M	M	CP	CN
Absorbance value (mean and standard deviation)	0.402 ± 0.0	0.384 ± 0.051	0.358 ± 0.067	0.353 ± 0.046	0.398 ± 0.051	2.163 ± 0.209	0.154 ± 0.028
Degree of reduction (%)	87.7	88.6	90.1	90.1	87.9	0.00	-
Absorbance value (mean and standard deviation) 5W	0.176 ± 0.052	0.172 ± 0.048	0.168 ± 0.051	0.161 ± 0.039	0.189 ± 0.044	2.163 ± 0.209	0.154 ± 0.028
Degree of reduction %)	98.9	99.1	99.3	99.7	98.3	0.00	-
CFU/mL resulting from cultivation (mean)	<102	<102	<102	<102	<102	2.6 ± 105	-
Degree of reduction % (log10)	>99.9 (3log)	>99.9 (3log)	>99.9 (3log)	>99.9 (3log)	>99.9 (3log)	0.00	-

Etanercept 2.5 μg/mL (E); Infliximab 50 μg/mL (I); Metotrexat 2.5 μg/mL (M); Etanercept 2.5 μg/mL + Metotrexat 2.5 μg/mL (E + M); Infliximab 50 μg/mL + Metotrexat 2.5 μg/mL (I + M).

1.9.3.4 *Discussion*

There is a growing awareness in the scientific community of the effect of the diversity and composition of the microbiota on a patient's response to synthetic and biological immunosuppressive therapy. Therapies such as cyclophosphamide and methotrexate (MTX) can induce a diffuse depletion of the intestinal microbiota, associated with the increase in commensal species in favor of pathogens that can damage the intestinal barrier, changing the permeability of epithelial cells, with consequent bacterial translocation. The risk of infections in patients with autoimmune diseases is higher than in healthy subjects, due to both endogenous factors (dysfunctional immune system) and exogenous factors (comorbidities and immunosuppressive therapy). Moreover, infections can induce relapses or cause a more severe clinical evolution, sometimes causing death in immunocompromised subjects (Picchianti-Diamanti et al., 2018). Immunosuppressive treatment is the main exogenous factor contributing to the increased risk of infections in such patients.

Anti-TNFα agents are an approved therapeutic line in the treatment of autoimmune diseases and, although these agents have demonstrated safety and a good efficacy profile, some patients may experience adverse effects related to treatment administration (Wysocki amd Paradowska-Gorycka 2022).

In our study, the growth of *A. actinomycetemcomitans* in vitro in the absence of any therapy was slightly inhibited by the drug combinations Etanercept 2.5 μg/mL + Methotrexate 2.5 μg/mL, and Infliximab 50 μg/mL + Methotrexate 2.5 μg/mL. In the absence of other studies in the literature that analyze in vitro the effect of these immunomodulating drugs on a strain of *A. actinomycetemcomitans*, we can assume that they act by inhibiting certain virulence factors that are expressed at the level of bacterial multiplication.

Because anti-TNF-α drug treatment is commonly used to control the inflammatory process, such therapy may also be relevant for the management of periodontitis. Some data in the literature suggest that epigenetic changes through regulating pro-inflammatory responses via

NFkB affecting TNF-α may be significantly involved in the pathogenesis of rheumatoid arthritis and other chronic inflammatory diseases (Zamri et al., 2020).

Taking into account the data from microbiological, immunological and histopathological studies that indicate that *A. actinomycetemcomitans* is important in the etiology of periodontal disease, being a potentially virulent bacterium including multiple immune evasion mechanisms, each with a crucial role in the conversion of the periodontal tissue to a pathological status, we can also take into account the hypothesis of the modulation by the pharmaceutical combinations used in this study and of the profoundly altered oxidative stress autoimmune diseases.

P. gingivalis and *A. actinomycetemcomitans* were observed to induce IL-17 production by CD4+ T cells when cultured with monocytes. *A. actinomycetemcomitans* induced IL-17 production and monocyte activation in a lower number when compared to *P. gingivalis*. The ability of cells stimulated with these periodontopathogens to produce IL-6, IL-1β, TNF-α and IL-23 may contribute to the induction of Th17 in situ. These authors concluded that *P. gingivalis* and *A. actinomycetemcomitans* can activate monocytes, the result being increased IL-17 production by CD4+ T cells in vitro (Cheng et al., 2016).

Photodynamic therapy and diode laser therapy are used as an adjunct to conventional periodontal therapy, not as replacements for it. These methods must be implemented after the mechanical disaggregation of the biofilm has been achieved, targeting the floating form of bacteria in the periodontal pocket. Although in vivo periodontal pathogens live in complex communities, either in the form of a biofilm or in a floating form, we chose an experimental model of bacterial monoculture due to the fact that it allows the investigation of variables under single, standardized, reproducible test conditions. This was also the reason why we chose to optimize within our in vitro experimental study the bacterial monocultures of *Aggregatibacter actinomycetemcomitans* in a planktonic state.

We evaluated the effect of laser and photodynamic therapies in rheumatoid arthritis patients in a previous study. Clinical periodontal measurements and oxidative stress markers (8-hydroxy-2′-deoxyguanosine (8-OHdG) and 4 hydroxynonenal (4-HNE)) were analyzed at baseline and 6 months after periodontal treatment and we observed that the analyzed oxidative stress markers decreased significantly following non-surgical periodontal therapy in both rheumatoid arthritis and systemically healthy patients. Moreover, the association of laser therapy with scaling and root planing and photodisinfection with scaling and root planing yielded the best clinical and paraclinical outcomes when compared to classical periodontal therapy alone.

Ultraviolet (UV) light has been used as phototherapy for various skin diseases, such as psoriasis, atopic dermatitis, vitiligo and photodermatoses. The wavelengths of UV light are classified as UVC (100–280 nm), UVB (280–315 nm) and UVA (315–400 nm). UVA and UVB radiation have often been used to treat dermatoses. UVB light irradiation has been reported to alter T regulatory (Treg) cells and exhibit immunosuppressive effects in patients with psoriasis and atopic dermatitis (Ibbotson et al., 2018).

Therefore, UVB is effective in treating various skin diseases mediated by immunomodulation and could also be useful for the treatment of oral alterations such as

periodontal disease. Takada et al. demonstrated in 2017 that irradiation with 310 nm UV-LED radiation had weak bactericidal effects on oral bacteria, but showed low toxicity on gingival epithelial cells; moreover, irradiation induced the production of ROS and was particularly harmful to *P. gingivalis*.

These results suggest that the 310 nm UV-LED irradiation device may be useful for the treatment of periodontal disease, but further research is needed to evaluate the benefits and precise indications of this therapy.

It has been shown that 310 nm UVB irradiation induces an immunosuppressive reaction in the skin, causing an increase in IL-10 production, a decrease in the number of Th1 cells and an increase in Th2 cells (Elnazar et al., 2015). Immune hyperreactivity has been associated with the pathogenesis of chronic periodontal disease and the relationships between these cytokines, T cells and periodontal disease has been reported (Ebersole et al., 2017). Thus, the immunosuppressive reaction induced by UVB irradiation can be effective in the treatment of chronic periodontal disease.

UVC radiation has the strongest bactericidal effects and, in particular, around 254 nm, UVC is mainly absorbed in the DNA. UVC-LED radiation of 265 nm has a stronger bactericidal effect than UVB-LED irradiation. UVB irradiation also shows DNA damage, with the generation of pyrimidine dimers, but the effect is negligible. Therefore, UVB irradiation induces only low toxicity on gingival epithelial cells. Periodontal pathogenic bacteria such as *P. gingivalis* are anaerobic bacteria and are sensitive to reactive oxygen species; conversely, other oral resident bacteria, such as streptococci, are tolerant to reactive oxygen species (Takada et al., 2017). Therefore, reactive oxygen species induced by irradiation with a UVB-LED can selectively annihilate periodontopathogenic bacteria and cause the oral bacterial flora to change from periodontopathogenic to non-periodontopathogenic.

Blue LED light sources are commonly used in dentistry for the photopolymerization of dental resins. *A. actinomycetemcomitans* was inactivated at a rate exceeding five log10 steps of CFU after a 120 s irradiation with blue light derived from a dental unit (LED); however, when reducing the irradiation time to 20 and 40 s, the inactivation of the microbiological agent was ≤1 and 2 log10 steps, respectively. An effect due to illumination-induced heat in the samples can be neglected, as the temperature increase was only marginal (about 3 °C after illumination for 120 s). The blue light had no bactericidal effect on *E. coli*, which was chosen as a control because it has similar properties to *A. actinomycetemcomitans* and because its use as a negative control was already demonstrated in blue light phototoxicity experiments (Cieplik et al., 2014).

Photodisinfection therapy was tested by Oruba et al. in 2017 in an in vitro study that analyzed the effectiveness of this method using various operating parameters for the eradication of periodontopathogens. The conclusions of the study were that bacteria differ in their susceptibility to photodynamic inactivation, namely, an eradication of *P. gingivalis* and an inhibition of *F. nucleatum* was obtained, but the effect was not the same for *A. actinomycetemcomitans*, which proved to be insensitive to such therapeutic applications (Oruba et al., 2017).

In our study, a degree of reduction >99.9 (3log) was obtained for photodisinfection therapy with a photosensitizer based on methylene blue, after a 30 s exposure to light radiation, regardless of the drug combinations used.

Another study investigated the effect of methylene blue-mediated antimicrobial photodynamic therapy (PDT) on cell viability and expression of the fimbriae-associated gene (rcpA) in *A. actinomycetemcomitans*. To determine the dose-dependent effects of PDT, a strain of *A. actinomycetemcomitans* (ATCC 33384) was irradiated after exposure to methylene blue, followed by cell survival analysis and expression ratio of rcpA by CFU and real-time PCR testing. In that study, the administration of 100 μg/mL methylene blue caused a significant reduction in the growth of *A. actinomycetemcomitans* compared to the control ($p < 0.05$).

The sub-lethal dose of PDT against *A. actinomycetemcomitans* was 25 μg/mL at a fluence of 93.75 J/cm^2. The sub-lethal dose of PDT could lead to an approximately fourfold suppression of rcpA expression, thus significantly reducing the expression of rcpA as an important virulence factor of this strain in cells, and, therefore, PDT may be a valuable tool for the treatment of *A. actinomycetemcomitans* infections (Pourhajibagher et al., 2017).

Fekrazad et al. (2017) exposed cultures of *A. actinomycetemcomitans* to a 662 nm laser in the presence of the photosensitizer Radachlorin® and to an 810 nm laser in the presence of the photosensitizer EmunDo®, then the bacterial suspension of each well in the study groups was diluted and subcultured on the surface of Muller–Hinton agar plates, with CFU analysis. *A. actinomycetemcomitans* suspensions showed a significant reduction in the case of both therapies, being thus recommended by the authors as a promising new approach in neutralizing periodonto-pathogenic bacteria.

Another study, this time performed on biofilm, concluded that, separately, a photoactivator based on methylene blue and light radiation cannot induce the inactivation of *A. actinomycetemcomitans*. The authors also observed that photodisinfection is dependent on exposure time, with the highest bacterial reduction (99.85%, $p = 0.0004$) occurring after exposure to methylene blue and photodynamic for 5 min, and for these parameters, the biofilm also suffered important structural damage (Alvarenga et al., 2015).

The efforts to find new photosensitizing agents are evident in the literature, with two in vitro studies from 2018 proposing a curcuma extract with a 450 nm light source to be used for this purpose. Both studies concluded that the toxic effect on *A. actinomycetemcomitans* was dose-dependent and that this substance could be used as a photosensitizing agent in periodontal disease therapy (Saitawee et al., 2018, 33. Pourhajibagher et al., 2018).

Zirconia implants were contaminated with a bacterial suspension of *Prevotella intermedia*, *Actinomyces actinomycetemcomitans* and *Porphyromonas gingivalis* and then randomly assigned to four groups according to the decontamination protocol: Group 1 (PDT1)-PDT (660 nm, 100 mW) with toluidine blue; Group 2 (PDT2)-PDT (660 nm, 100 mW) with phenothiazine chloride; Group 3 (LAD)–LED device with toluidine blue; and Group 4 (TB)-toluidine blue without the application of light. The analysis recorded in all study groups significant reductions in the number of CFU compared to controls ($p < 0.05$) and PDT1, PDT2 and LAD had the highest

bacterial reduction with respect to each separate bacterial species but also with respect to the total number of bacteria.

The SEM analysis of the implant surfaces did not show any changes after the treatment procedures, thus demonstrating high effectiveness in the decontamination of zirconium dental implants (Azizi et al., 2018). Thus, the indications of photodynamic therapy could also be extended in the treatment of peri-implantitis, a pathology that is difficult to treat and which has serious consequences on the oral cavity.

For the diode laser therapy, the most significant reductions in the amount of *A. actinomycetemcomitans* were obtained when irradiating with a power of 5 W, the percentages being >98% regardless of the pharmaceutical associations. The laser we use has a wavelength of 940 nm, which is in the infrared spectrum. We chose this wavelength because in this particular interval, there is a maximum absorption of melanin, hemoglobin and water, compared to other parts of the infrared spectrum, so we can obtain a maximum antimicrobial effect but at the same time a protection of the host cells.

A study observed that the density of power did not modify the photosensitizers' absorption of light. *A. actinomycetemcomitans* was inactivated completely through the association of proper photosensitizer absorption and irradiation characteristics (de Sousa et al., 2022).

A systematic review analyzed a total of 32 in vitro studies, among those, 25 used in-suspension microorganisms and observed a reduction greater than or equal to 3 logs CFU/mL of periodontopathogens. In biofilms, three studies highlighted showed a reduction equal to or greater than 3 logs CFU/mL. Nonetheless, the authors stress the importance of light parameters standardization, photosensitizer type and pre-irradiation time prior to performing clinical studies (Sales et al., 2022).

However, other authors stress the inefficacy of one session of photodisinfection, concluding that solely one application of this technique in adjunction to non-surgical periodontal treatment is inefficient in *P. gingivalis* and *A. actinomycetemcomitans* positive periodontitis patients (Aabed et al., 2022).

Recent studies have tested biofilms of *A. actinomycetemcomitans* plus *Streptococcus sanguinis* grown on bovine root surfaces, treated with an 810 nm diode laser, pulsed mode, non-contact, with an interval and pulse length of 20 ms. Four protocols were tested, namely, one episode of 1.5 or 2.5 W for 30 s and three episodes of 1.5 or 2.5 W for 30 s. The authors used as negative control the absence of any treatment and 0.2% chlorhexidine as positive control. Chlorhexidine treatment and all laser protocols except for that using a single episode of 1.5 W reduced the number of *A. actinomycetemcomitans* in either the single-species or the dual-species biofilm compared to the negative control. The authors concluded that a higher percentage of *A. actinomycetemcomitans* reduction occurred after increasing the power or repeating the irradiation, but they failed to eradicate the biofilm regardless of the applied protocol (Tantivitayakul et al., 2018).

Analysis of the antimicrobial efficiency of irradiation with light from the visible spectrum on oral bacteria in vitro, through the evaluation of an impressive number of studies that took into account various oral pathogens (incriminated particularly in periodontitis) such

as *Fusobacterium* spp., *Porphyromonas* spp., *Aggregatibacter* spp., *Prevotella* spp., *Staphylococ cus* spp., *Streptococcus* spp., showed that laser irradiation could be a viable option for controlling *A. actinomycetemcommitans* infection, but for best results light sources with the absorption spectrum for flavins and porphyrins should be used; practically, it appears that the eradication of bacteria in planktonic cultures is especially effective in the case of black-pigmented ones, such as *Porphyromonas* and *Prevotella* spp. Regarding bacteria organized in biofilms, the reported evidence is less clear, and more studies that take into account multiple working protocols are needed (Pummer et al., 2017).

A study found that *A. actinomycetemcomitans* initiates neutrophil-mediated leukotoxin A (LtxA) hypercitrullination, being detected in the oral microbiome of rheumatoid arthritis patients, where it could act as a bacterial trigger of the disease (Konig et al. 2016). The development of therapeutic strategies targeting this bacterium could prevent the onset, or improve the course of, rheumatoid arthritis developed through this etiopathogenic pathway.

Overall, the analyzed results imply that laser and photodynamic therapy could be a treatment option for the eradication of *A. actinomycetemcommitans*. However, additional studies are needed, especially regarding the existence of the biofilm, as very likely *A. actinomycetemcomitans* is not the only microorganism capable of inducing hypercitrullination in neutrophils.

Photodisinfection and diode laser therapy could be an effective option for antimicrobial applications in dentistry, for example, in the treatment of periodontal disease and peri-implantitis, given that blue light irradiation has also been shown to be effective against other periodontal pathogens such as *Porphyromonas gingivalis*, *F. nucleatum* and *Prevotella* spp. However, further studies are necessary (Salvi et al., 2020, Choe et al., 2021).

Limitations of this study consist of the inherent limitations of an in vitro study, in that the conclusions need to be extrapolated with care to the clinical implications. In this study, we only analyzed the effectiveness of a diode laser and photodisinfection on a single bacterial strain of *A. actinomycetemcommitans*; however, the oral environment is much more complex.

In future studies, we will expand our research to other immunomodulatory medications and their effect on other periodonto-pathogens.

1.9.3.5 *Final remarks*

The association of a conventional antirheumatic drug with anti-TNF-α therapy determined a significantly greater inhibition of a type strain of *A. actinomycetemcomitans* compared to monotherapy, in vitro.

Photodisinfection caused a significant reduction in a type strain of *A. actinomycetemcomitans* after a 30 s exposure in vitro, regardless of the pharmaceutical associations of biological and conventional DMARDs.Irradiation with a diode laser for 30 s at a power of 5 W caused a greater reduction in *A. actinomycetemcomitans* compared to irradiation with 1 W.

Our in vitro study did not record statistically significant differences between the eradication of *A. actinomycetemcomitans* by photodynamic therapy with methylene blue and the diode laser

at a power of 5 W. Future studies should focus on identifying possible interactions between the applied methods in order to implement them successfully in standardized and optimized clinical protocols.

Although mainstream medicine has traditionally prioritized pharmacological and sur- gical treatment methods, full-spectrum light therapy has emerged as a credible alternative therapeutic option and is now being used in a variety of settings. In fact, there is currently promising evidence for the use of light therapy across a spectrum of oral hard and soft tissues and in a variety of important dental subspecialties, such as endodontics, periodontics, orthodontics, and maxillofacial surgery.

The merging of diagnostic and therapeutic light procedures is also seen as a promising area of future development. In the next decade, several light technologies are predicted to become integral components of the practice of modern dentistry. To conclude, photobiology plays a crucial role in changing paradigms regarding optimizing or implementing new clinical protocols in contemporary dental medicine.

1.9.4. New approach in periodontal disease

1.9.4.1 *Introduction*

Periodontitis is an inflammatory disease of the tissues that serve to maintain and sustain the functionality of the teeth on the dental arches. Its etiology is often multifactorial, the main cause being the onset of periodontal dysbiosis, in favour of anaerobic gram-negative bacteria (Van Dyke et al., 2020). The human body will react to this dysbiosis through innate and adaptive defense mechanisms, with the manifestation of an inflammatory response, detectable at molecular and clinical levels. If the causal factors are not removed, the inflammation may evolve, turning initially reversible lesions into attachment losses, with the onset of periodontitis (Gibertoni et al., 2017).

A number of local and systemic factors can influence either the retention of periodonto pathogenic bacterial plaque, with changes in its quantity and quality, or the ability of the immune system to effectively counteract bacterial aggression (Zhao et al., 2022). The latter include diabetes mellitus (DM). This study focuses on type II DM patients with periodontitis; type II DM is a metabolic disease, often the consequence of an inadequate diet, which may be associated with other predisposing factors (tobacco use, alcohol, genetic factors, etc.) (Namayandeh et al., 2019).

The overall impact of DM is epidemic in nature, with worrying statistical parameters, especially in light of the complications that patients with diabetes may experience. These include changes of a micro- and macro-vascular nature, which make DM the leading cause of blindness (diabetic retinopathy), amputation of the lower limbs, kidney failure, hypertension or even death from cardiovascular causes (Eschwège 2000).

Periodontal disease has been identified as the sixth complication of DM, as a result of a combination of mechanisms that include reduced immune response capacity in the patient with diabetes, increased markers of inflammatory and oxidative stress through the production of advanced glycation end-products (AGEs) and tissue healing deficits (Loe 1993). Of course, the manifestations of these complications are all the more important as the metabolic control is more

deficient (Vijan et al., 1997). A measure of this ability is the evaluation of glycated hemoglobin (HbA1c). Moreover, it seems that peri- odontal inflammatory status can also negatively influence metabolic control in patients with diabetes (Astolfi et al., 2022).

The treatment of the patient with periodontal impairment includes, first of all, the removal of the risk factors, which, in the first stage, involves, in addition to the patient's motivation and awareness, professional hygiene techniques (Elkerbout et al., 2022). Scaling and root planing (SRP) remain the gold standard in periodontal therapy, with the aim of obtaining "plaque-free" coronal and root surfaces, with a relief that allows the creation of a new periodontal attachment. Nevertheless, the majority of clinical trials have suggested that non-surgical periodontal debridement improves glycemic control among individuals with type 2 diabetes mellitus (Koromantzos et al., 2011; Sun et al., 2011; Jain et al., 2019).

A meta-analysis performed by Jain et al. (2019) found that SRP treatment decreased HbA1c by 0.26% ($p = 0.17$) at 3–4 months compared to the control group. A systematic review and meta-analysis (Madianos and Koromantzos 2018) found that SRP generated a statistically significant reduction in HbA1C levels at 3 months of about 0.40% (range 0.27–0.65%).

However, it seems that SRP cannot completely remove bacterial species and their products, especially from periodontal soft tissues (Schulz et al., 2022). Thus, new methods of additional periodontal treatment have been developed, methods that involve local and/or systemic administration of antiseptic or antibiotic substances (Jepsen et al., 2016), therapies to modulate the host immune response (sub-antimicrobial doses of doxycycline) (Golub and Lee 2020) or photo-bio-modulation therapies and photo-disinfection of periodontal pockets (Sobotta et al., 2019).

Antimicrobial photodynamic therapy (aPDT) is an adjuvant, minimally invasive therapeutic method that involves the use of three main components: light source (laser or LED), photosensitizer and singlet oxygen released into the tissue, the latter generating the bactericidal effect (Sales et al., 2022). Most studies investigating the effects of aPDT in patients with periodontitis have used phenothiazine derivatives (methylene blue, toluidine blue), xanthene or riboflavin as photosensitizers. Recently, special attention has been paid to indocyanine green, as a product with photosensitizing potential (Sobotta et al., 2019).

Indocyanine green consists of two aromatic parts linked together by a polyunsaturated chain; it is a dye commonly used in medicine, especially in imaging investigations (Qi et al., 20121). It penetrates rapidly into tissues and has low toxicity, being approved by the FDA for clinical use and recognized as non-toxic (Porcu et al., 2016). The absorption range for indocyanine green is between 600 nm and 900 nm and it emits fluorescence between 750 nm and 950 nm (Houthoofd et al., 2020).

Data on the efficacy of this substance in patients with periodontitis and DM are lim- ited; for this reason, we propose a study aimed at investigating the effects on periodontal clinical parameters and HbA1c of aPDT adjunctive therapy with indocyanine green as a photosensitizer in patients with periodontitis and type II diabetes, compared to the use of SRP alone. Our primary outcome investigation regarded the local, periodontal response to treatment of a particular group

of subjects, prone to severe periodontal destructions and impaired healing. As a secondary outcome, we wanted to assess any potential supplementary benefits other than those already demonstrated by scaling and root planing, on HbA1c.

The proposed null hypothesis was that aPDT with indocyanine green does not provide any additional benefits to SRP on local periodontal parameters, nor on glycemic control, measured by HbA1c, for this particular category of patients.

1.9.4.2 *Materials and Methods*

Patient Selection

This prospective, randomized controlled, single-blind interventional study was performed on 49 patients diagnosed with periodontitis and type II diabetes. The methodology of the study was in accordance with the rules set out in the Declaration of Helsinki and was approved by the Bioethics Commission of the institution. All study participants signed the informed consent form and were aware of their right to withdraw from the study at any stage of the study without being subject to any sanctions.

The study included male and female subjects, diagnosed with type II diabetes mellitus by the diabetologist, and who presented periodontal probing depths higher than 5 mm upon periodontal examination. Exclusion criteria were represented by: (i) systemic diseases other than diabetes, which could influence periodontal status; (ii) smoking; (iii) history of antibiotic therapy, anti-inflammatory therapy or periodontal treatment in the last 3 months; (iv) significant changes in DM treatment during the study period.

Sample Size Calculation and Randomization

Based on previous findings (Zhang et al., 2013), a probing depth (PD) reduction of 1 mm was used to determine the size of the groups, with a power of 90%, and alpha set to 0.05, resulting in an optimal size of 22 subjects per group. However, in order to counteract potential withdrawals from the study, we set a size of 25 patients per group, estimating an abandonment rate of 10%. The 50 resulting subjects were, thus, divided into two groups: patients who followed scaling and root planing therapy (n = 25) (SRP group) and patients who followed SRP and aPDT therapy with diode laser and indocyanine green (n = 25) (SRP + aPDT group); one patient who did not attend all aPDT sessions was finally excluded from the study group.

The SRP group consisted of 56.00% male subjects and 44.00% female subjects, with an overall mean age of 54.24 ± 3.41 years old; the SRP + aPDT group consisted of 54.20% male subjects and 45.80% female subjects, with an overall mean age of 55.58 ± 3.62 years old. There were no significant differences in age between the two groups (p = 0.734) (Table I.3.4).

The distribution of patients to one of the two groups was randomized, through a system of sealed envelopes. Participants were offered randomly generated treatment assignments in sealed opaque envelopes, doubled with carbon paper, the therapeutic operator not being involved in making the envelopes.

Table I.3.4. Demographic parameters for study groups at baseline and after 6 months.

Parameter		SRP Group	SRP + aPDT Group
Subjects number (n)		25	24
Number of sites		474	445
Age (years) (mean±standard deviation)		55.24 ± 3.41	55.58 ± 3.62
Gender n (%)	**Male**	14 (56.00%)	13 (54.16%)
	Female	11 (44.00%)	11 (45.84%)

Clinical Investigations

For each patient included in the study, the following periodontal parameters were determined: (a) the simplified plaque index (PI) (O'Leary et al., 1972) with the qualitative percentage determination of the surfaces with bacterial plaque; (b) bleeding on probing index (BOP), with the qualitative determination of the percentage of bleeding sites following the periodontal probing; (c) probing depth (PD), established by inserting the periodontal probe into the periodontal pocket, evaluated at six points per tooth (mesial–buccal, buccal, distal–buccal, mesial–lingual, lingual, distal–lingual); (d) periodontal clinical attachment loss (CAL), as- sessed as the distance between the enamel–cement junction and the base of the periodontal pocket. A North Carolina No.15 Periodontal Probe (Hu-Friedy, Chicago, IL, USA) was used for clinical measurements.

All periodontal assessments were performed by an experienced qualified examiner. Calibration was accepted if >90% of the measurements were reproduced within 48 h. Clinical examinations were performed blind to the type of treatment followed by the subjects. Clinical measurements were performed at baseline (T0) and 6 months apart (T1).

Evaluation of HbA1c

For the analysis of glycated hemoglobin, venous blood was collected in 3 mL vacu- tainer with EDTA K3 (FL Medical, Torreglia PD, Italy). The quantification of glycated hemoglobin in total hemolyzed blood was based on a turbidimetric inhibition reaction, as previously described (Anton et al., 2021; Zaharescu et al., 2021). Glycated hemoglobin A1c (HbA1c) was determined for each patient at baseline and after 6 months. The method of determining HbA1c was immunotur- bidimetric (Boehringer Mannheim, Baden-Wurttemberg, Germany), a test characterized by high specificity for anti-HbA1c antibodies (Hamwi et al., 1995).

Treatment Methods

All patients in the SRP and SRP + aPDT groups underwent non-surgical periodontal treatment, which involved ultrasound scaling (Woodpecker UDS-A-LED, Guilin Wood- pecker Medical Instrument Co., Ltd., Guangxi, China) and root planing (Gracey Standard and Mini—Hu-Friedy, Chicago, IL, USA) (SRP), in one session. All patients were instructed in the technique of brushing their teeth and to avoid rinsing with antiseptics during the study.

Patients in the study group also received aPDT therapy with diode laser and indocya- nine green. For the preparation of the photosensitizer, the indocyanine green powder (ICG Pulsion, PULSION Medical Systems SE, Feldkirchen, Germany) was mixed with pure water, with a ratio of 5 mL water to 25 g bottle of powder, to obtain a concentration of 5 mg/mL according to the

manufacturer's instructions. Since the aqueous solution of indocyanine green is unstable and must be used within 24 h, the fresh solution was prepared whenever necessary and the excess was discarded.

The laser system used in the present study was a diode laser (A.R.C. Laser GmbH, Nuremberg, Germany) with a wavelength of 810 nm. Based on previous findings 9Raut et al., 2018, Sethi et al., 2019), the laser was applied continuously with a power of 0.2 W and a total energy of 12 J. In the periodontal pocket 1 mL of indocyanine green solution was inserted with a blunt needle and left in place for 60 s. The pocket was immediately irradiated with a diode laser by placing the tip in the pocket and moving it circumferentially around the tooth for 60 s. The procedure was repeated at 7, 14 and 21 days.

All the patients were instructed to follow the nutritional recommendations and to continue their normal physical activity throughout the study. The flowchart of the study from enrollment to completion is presented in Figure I.3.5.

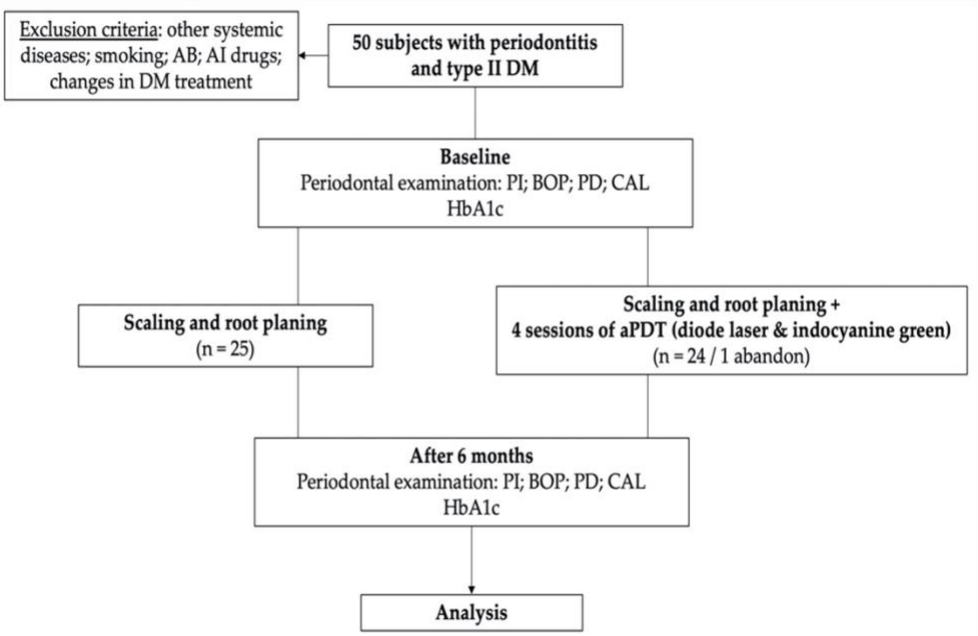


Figure I.3.5. The flowchart of the study. Figure 1. The flowchart of the study.

Statistical Analysis

All data were recorded in individual patient records, stored and statistically analyzed. For statistical analysis we used Microsoft Excel 2021 software (Microsoft, Washington, DC, USA) and Wizard 2 for Mac (Evan Miller®). The Shapiro–Wilk test was performed to determine the normality of the data distribution. The normally distributed values were compared with the t-Test and for the abnormally distributed values we used the Mann– Whitney test. The significance level was set at $p < 0.05$. The Pearson correlation test was used to determine the relationship between clinical parameters and HbA1c.

1.9.4.3 *Results*

The present study was completed in a SRP group of 25 subjects (474 sites, with a mean of 18.96 ± 4.48) who followed only SRP and a SRP + aPDT group of 24 subjects (445 sites, with a mean of 18.54 ± 4.07) that followed SRP + aPDT (Table I.3.4.).

At baseline, the PI values in the SRP group were 79.44 ± 6.31 and 80.04 ± 5.90 for the SRP + aPDT group, with no statistically significant difference ($p = 0.732$). After 6 months, PI showed significant decreases for both the SRP and the SRP + aPDT groups (17.72 ± 6.38, $p < 0.001$ and 17.08 ± 5.14, $p < 0.001$, respectively), with no statistically significant difference between groups at +6 months either (Table I.3.5.).

For BOP, the values at baseline were of 67.76 ± 6.57 in the SRP alone subjects and 68.67 ± 6.10 in the SRP + aPDT subjects, with a $p = 0.620$. The assessments after 6 months showed significant decreases for both groups: 8.08 ± 5.09 for the SRP group ($p < 0.001$) and 4.21 ± 3.85 for the SRP + aPDT group ($p < 0.001$); moreover, the decrease was more significant for the SRP + aPDT subjects ($p < 0.001$) (Table I.3.5.).

Table I.3.5. Clinical parameters for study groups at baseline and after 6 months.

Parameter	SRP Group (n = 25)			SRP + aPDT Group (n = 24)		
	Baselin *	+6 Month *	Δ #	Baselin *	+6 Months *	Δ #
PI	79.44 ± 6.31	17.72 ± 6.38 [a]	62 (60–66)	80.04 ± 5.90	17.08 ± 5.14 [a]	63 (60–67)
BOP	67.76 ± 6.57	8.08 ± 5.09 [a]	60 (55–66)	68.67 ± 6.10	4.21 ± 3.85 [ab]	65 [c] (55–69)
PD (mm)	5.54 ± 0.24	4.10 ± 0.22 [a]	1.4 (1.4–1.5)	5.53 ± 0.24	3.56 ± 0.19 [ab]	1.9 [c] (1.8–2.2)
CAL (mm)	4.51 ± 0.20	3.15 ± 0.17 [a]	1.40 (1.20–1.50)	4.50 ± 0.22	2.58 ± 0.19 [ab]	1.95 [c] (1.70–2.10)

Δ: level of decrease between evaluations; PI: Plaque Index; BOP: Bleeding on Probing Index; PD: probing depth; CAL: clinical attachment loss; * Values are expressed as Mean ± Standard Deviation; # Values are expressed as Median (Min–Max); a Intra-group $p < 0.05$ after 6 months (t-Test); b Inter-group $p < 0.05$ at the same time of evaluation (t-Test); c $p < 0.001$ (Man-Whitney test).

PD and CAL followed the same trend as BOP. At baseline, the PD and CAL values for the SRP group were of 5.54 ± 0.24 mm and 4.51 ± 0.20 mm, respectively; the values in the SRP + aPDT group were of 5.53 ± 0.24 mm and 4.50 ± 0.22 mm, with no statistically significant differences between the groups ($p = 0.878$ and $p = 0.951$, respectively). After 6 months, both parameters showed significant decreases; for the SRP group PD = 4.10 ± 0.22 mm, $p < 0.001$ and CAL = 3.15 ± 0.17 mm, $p < 0.001$; for the SRP + aPDT group, PD = 3.56 ± 0.19 mm, $p < 0.001$ and CAL = 2.58 ± 0.19 mm, $p < 0.001$. The decreases for PD and CAL were more significant in the subjects with SRP + aPDT (both p-values were lower than 0.001) (Table I.3.5.).

The values of HbA1c at baseline were of 6.52 ± 0.25 for the SRP group subjects and of 6.53 ± 0.24 for the SRP + aPDT group ($p = 0.900$). Both treatment options generated significant

and similar reductions of HbA1c for both groups. The HbA1c value for SRP group subjects after 6 months was 6.28 ± 0.24, p = 0.001 (Figure I.3.6.); the value for the SRP + aPDT group was 6.262 ± 0.040, p < 0.001 (Figure I.3.7.) and Δ values of 0.244 ± 0.014 for the SRP alone and of 0.26 ± 0.19 for the SRP + aPDT.

We found a strong positive correlation between HbA1c and tissue loss parameters (PD and CAL) in both groups. In the SRP group, we observed a ρ = 0.922 and ρ = 0.896 for PD at baseline and +6 months, respectively; for CAL the values were ρ = 0.796 and ρ = 0.809; also, the decrease in HbA1c (ΔHbA1c) correlated with ΔPD and ΔCAL (ρ = 0.773 and ρ = 0.491, respectively). In the SRP + aPDT group, the coefficients were ρ = 0.989 and ρ = 0.995 for PD at baseline and +6 months, respectively; for CAL the values were ρ = 0.918 and ρ = 0.794; also, the decrease in HbA1c (ΔHbA1c) correlated with ΔPD and ΔCAL (ρ = 0.963 and ρ = 0.697, respectively).

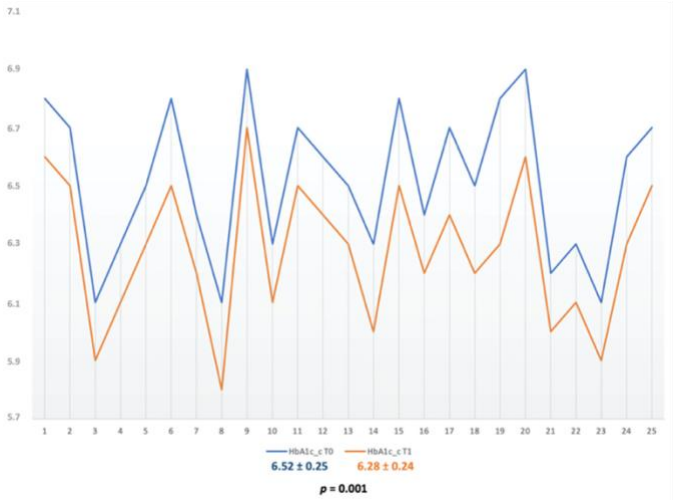


Figure I.3.6. HbA1c variations in the SRP group.

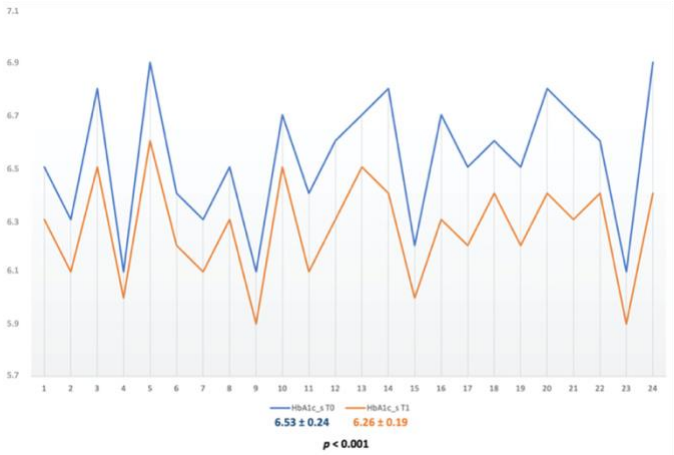


Figure I.3.6.HbA1c variations in the SRP + aPDT group

The treatment regime was well tolerated, without any significant side effects. Two patients in the SRP + aPDT group (8.33%) reported slight discomfort due to the local heating during aPDT.

1.9.4.4 *Discussion*

The present research proposed an evaluation of the effects of an 810 nm wavelength diode laser and indocyanine green supplementary therapy on periodontal clinical parameters and HbA1c, compared to SRP alone, in patients with diabetes mellitus and periodontitis. As far as we know, this is one of the first studies to investigate the effects of indocyanine green aPDT in patients with DM.

Photodynamic therapy has emerged as an additional method for periodontal treatment, an alternative to antibiotic therapy, which can cause a number of side effects, such as, in addition to toxicity, the emergence of resistant microorganisms. Existing data in the literature on the effectiveness of aPDT with various photosensitizers are heterogeneous. While some results suggest that the benefits of SRP supplementation with aPDT are minor (Sgolastra et al., 2013), other information, focused on the effects of aPDT with indocyanine green, would indicate that indocyanine green is more effective than previously analyzed agents in the management of periodontitis (Bashir et al., 2021). Although the local effects of using methylene blue or toluidine have been beneficial in terms of periodontal status improvements in patients with DM, they may generate a number of less desirable effects, such as tooth staining due to their prolonged adhesive property (Nagahara et al., 2013).

The adjunct aPDT therapy, with various photosensitizers, was previously investigated in DM subjects; the data suggest that, even if the clinical periodontal improvements were significant when compared to SRP alone, the HbA1c reduction as additional benefit were modest. Nevertheless, the found studies had significant heterogeneity regarding the energy source, the used laser power, irradiation sessions and also concentrations for photosensitizers (Al-Zahrani et al., 2009; Macedo et al., 2014; Ramos et al., 2015; Dos Santos et al., 2016).

Indocyanine green is considered a safe substance without the side effects of antibiotics . Mechanisms that could explain the beneficial effects of indocyanine green as an adjunct to aPDT have been investigated in clinical, microbiological, and immunological studies. The hypotheses investigated include the bactericidal effect on periodontal pathogens, as well as potential effects on the local immune response (Bashir et al., 2021).

The mechanism of action of indocyanine green is different from other photosensitizers—it exhibits a 20% photodynamic effect and the main action is through photothermal effect, which induce cell damage by increasing intracellular temperature [36]. Photothermal therapy involves the energy absorption from laser radiation by indocyanine green, inducing an effectively elevated local temperature (Sukumar et al., 2020).

In vitro studies showed that indocyanine green aPDT could effectively reduce bacterial load in periodontal pockets (Sukumar et al., 2020). Boehm and Ciancio (2011) demonstrated that indocyanine green aPDT generated a significant killing of A. actinomycetemcomitans and P. gingivalis. Srikant et al. (2015) observed substantial reduction in the proportion of viable bacteria

at the end of 1 week in sites receiving indocyanine green (5 mg/mL) aPDT compared to sites which followed SRP alone or SRP plus low-level laser therapy.

An investigation found that, in addition to photothermic effects, indocyanine green was shown to have a photodynamic effect by generating reactive oxygen species (ROS) (You et al., 2017). Since pathogens indicate various susceptibility for singlet oxygen and radical species, it is important for future studies to measure the ROS under the selected experimental conditions. This would lead to understanding the photoreaction mechanisms and, consequently, mechanism of the applied aPDT.

Our study investigated the effects of additional aPDT with indocyanine green to SRP versus SRP alone on periodontal clinical parameters in patients with type II diabetes and periodontitis: bacterial plaque index (PI), probing bleeding index (BOP), probing depth (PD) and loss of periodontal clinical attachment (CAL), along with HbA1c analysis, assessments performed at baseline and after six months. It is important to note that there were no statistically significant differences between the groups regarding all these parameters at baseline.

At the six-month evaluations, we noticed statistically significant differences compared to baseline for all clinical parameters in both groups. Following comparisons between groups at six months, we noticed that there were no significant differences between PI values, unlike the studies conducted by Sethi and Raut (2019) or Vangipuram et al. (2021), where PI was significantly lower in the SRP + aPDT group than in the SRP group.

We specify that in the study there was a rigorous protocol of motivation and awareness of the patient, the brushing technique being clearly explained, with the reinstatement of the instruction whenever necessary. We also cannot ignore the fact that patients may have shown high compliance as a result of awareness of participation in a study (potential Hawthorne effect) (McCarney et al., 2007). We also recommended that patients avoid oral rinses with antiseptics during the study to avoid the risk of bias.

Moreover, in a systematic review and meta-analysis it was suggested that multiple applications of aPDT are more efficient in reducing periodontal pathogens compared to a single application (Jervoe -Storm et al., 2015). Based on these findings, our treatment protocol involved four sessions of aPDT.

Statistically significant differences between groups appeared when comparing BOP, PD and CAL; subjects who followed SRP + aPDT showed more significant decreases than subjects who followed only scaling and root planing. These data are consistent with the results of other studies. Al-Momani (2021), in a split-mouth design study in patients with type II diabetes and stage III periodontitis, grade C, noticed significant improvements in clinical and antimicrobial parameters. Raut et al. (2018) also observed a reduction in PD and CAL in the test group with indocyanine green aPDT therapy compared to the control group after 6 months.

A recent meta-analysis observed statistically significant improvements in aPDT results with indocyanine green at 3 months and 6 months after therapy, compared with single SRP; PD demonstrated an average additional reduction of 1.17 mm and 1.06 mm at 3 and 6 months, respectively. For CAL, an average additional gain of 0.70 mm and 1.03 mm was observed at 3 and 6 months, respectively (Bashir et al., 2021).

Highly significant reductions for the indocyanine green aPDT group were also observed for BOP and PD by Monzavi et al. (2016) and Hill et al. (2019), without significant benefits, however, for CAL. It is worth noting, however, that they used a concentration of indocyanine green solution of 1 mg/mL. Similar to our study, Shingnapurkar et al. (2016), using the same concentration of 5 mg/mL indocyanine green, observed significant reductions for CAL, in addition to BOP and PD.

Bassir et al. (2013), in a study on aPDT with indocyanine green 1 mg/mL, for 30 s per session, for four sessions in patients with periodontitis, concluded that indocyanine green with an 810 nm diode laser in combination with SRP led to complete resolution of inflammation and significant reduction in periodontal pocket.

One study compared the antimicrobial efficacy of aPDT with indocyanine green, metronidazole gel, and chlorhexidine gel in vitro and reported that all of these modalities significantly reduced bacterial load (Fekrazad et al., 2017). However, the major clinical disadvantages associated with chlorhexidine, including taste change and pigmentation of teeth and mucosa, were absent in the case of aPDT. In addition, Chiang et al. (2020) demonstrated that cytotoxicity on oral cells by aPDT with indocyanine green was significantly less prominent compared to that of chlorhexidine.

Moreover, the subgingival environment is characterized by lack of oxygen, which may not provide favorable conditions for better action of these traditional photosensitizers, while indocyanine green works even in the absence of oxygen. Importantly, indocyanine green gets stimulated only in the presence of laser light, hence only the target (bacterial cells) gets affected in a dose-controlled fashion.

Thus, there is a need to establish a standard protocol for the use of indocyanine green solution in periodontal therapy of aPDT, in terms of solution concentration, but also in the number of aPDT sessions. During the studies, including the present study, which used four sessions of aPDT, no adverse effects were reported to contraindicate the repetitive application of aPDT with indocyanine green. Two patients reported mild discomfort during aPDT application but did not require discontinuation of therapy.

HbA1c followed significant decreases in both subjects who had only scaling and root planing, and those with SRP + aPDT (0.244 ± 0.014 for the SRP alone and 0.267 ± 0.02 for the SRP + aPDT). This aspect is consistent with other data in the literature—Jain et al. observed, in a meta-analysis, a decrease in HbA1c by 0.26% ($p = 0.17$) at 3–4 months after SRP. This reduction can be considered, from a global point of view, as minor; further investigations, with full apprehension of all patient variables, are required in order to determine if this reduction can be based on the effects of the local, periodontal treatment.

Nevertheless, even if minor, reductions of HbA1c can exert a considerable impact on the systemic status of the patient with type II diabetes, especially on its complications. Each 1% reduction in the mean HbA1c was associated with a 21% reduction in risk for diabetes-related deaths, 14% for myocardial infarction and 37% for microvascular complications (Stratton et al., 2000). What we noticed, however, in this study is that aPDT did not generate more significant

effects on HbA1c than SRP alone. Thus, we can note that the null hypothesis was partially refuted; aPDT with indocyanine green had more statistically significant effects on BOP, PD and CAL, but not on PI and HbA1c.

Therefore, this type of adjunctive therapy has the potential to generate supplementary clinical benefits to SRP on the periodontal destructive status of patients with type II diabetes, with no significant benefit to SRP in terms of glycemic control.

Of course, this study also has a number of limitations. Further investigations of our proposed therapeutic protocol on larger groups of DM and periodontitis subjects are required, with the inclusion of systemically healthy subjects as controls. We also did not assess the duration of diabetes illness, a factor which can negatively impact the evolution of periodontitis and the response to periodontal treatment (Kim et al., 2013). Even if the exact DM treatment variables were not investigated in detail, any changes in DM treatment or diet were considered as exclusion criteria.

Moreover, our study was predominantly clinical; we intend to continue and expand our investigations of the microbiological and molecular changes that could be generated by aPDT periodontal adjunctive treatment with indocyanine green in type II DM patients. Interesting observations might also emerge from comparative studies with other available photosensitizers, such as methylene blue, toluidine blue or curcumin. Moreover, further research could investigate the potential effects of this particular therapy in patients with other systemic conditions, such as osteo-articular, renal or cardiovascular diseases and periodontitis.

1.9.4.5 *Final remarks*

Within the limitations of our study, the therapeutic protocol of four sessions with an 810 nm wavelength diode laser and 5 mg/mL indocyanine green as adjunctive to scaling and root planing resulted in statistically higher reductions in bleeding on probing, probing depth, and periodontal clinical attachment loss in patients with type II diabetes mellitus and periodontitis, when compared to SRP alone. Further investigations need to clarify the clinical and molecular advantages of using a photosensitizer that does not require the presence of oxygen in the microaerophilic deep periodontal pockets of DM patients.

SECTION II -
PERSPECTIVES – NEW RESEARCH DIRECTIONS

The experience and professional results so far have strengthened my capacities and skills in the field of analysis and synthesis, medical practice and an easy acquisition of new techniques in the fight against periodontal disease. Thus, I proposed, and became a follower of a modern, minimally invasive dentistry, which successfully replaces the scalpel and the equipment that, as a rule, cause anxiety and discomfort to the patient. The quality of the intervention and the results at a distance render it incomparable with the classic one. Recovery after laser is much faster, bleeding during laser-assisted interventions is almost non-existent, and edema is reduced. It is a modern field, with multiple challenges, but not impossible to complete successfully. It requires proper training and, of course, rigorous equipment, which is not cheap at all.

Advances in periodontal science and practice over the last decade have radically changed the understanding of periodontal diseases and have opened new, exciting prospects for both medical and surgical therapy of periodontal diseases. The establishment of the aetiology and pathogenesis of periodontitis, the understanding of the unique genetic and environmental susceptibility profile of affected subjects, and the recognition of the systemic implications of periodontal infections are the key research findings. The use of randomised, controlled, clinical trials has allowed the development of evidence-based periodontology. Adjunctive antimicrobial therapy, regenerative periodontal surgery, periodontal plastic surgery, bone regeneration surgery in the light of implant treatment, and advanced soft tissue management at implant sites have radically changed practice.

II .1. FUTURE DIRECTIONS IN MEDICAL DENTAL ACTIVITY

I have worked hard to successfully integrate clinical practise with the teaching act during my whole career as a dentist and university teacher. I have also used scientific research as a source of knowledge to support both the teaching act and the medical act.

I believed that continuing efforts to update previously learned information, through individual study and by taking part in national and worldwide scientific activities, were the foundation for professional progress. Once I connected with the beginner in the area, I made an effort to learn technical abilities so I could use them in both my daily work and my theoretical and practical teaching.

In terms of innovations, I'll make an attempt to remain up to speed on the most recent advancements in periodontology so that I may update both my diagnostic and therapeutic procedures in accordance with general trends. Additionally, in an effort to broaden my views, I will concentrate my training in those areas that I view as weak points.

II.2. FUTURE DIRECTIONS IN RESEARCH ACTIVITY

II.2.1. PREVENTION

Despite the global scope of periodontal disease, its impact on pain, oral function, and the wellbeing of individuals, and the disproportionate burden of disease and the socio-economic impact on communities, the perception that periodontal disease is a public health problem remains low. Although there have been substantial improvements in our understanding of the etiology of periodontal disease and how we can prevent and control it, these advances have been primarily focused on individual, patient-focused approaches.

The prevention of periodontal disease depends on improving currently available individual interventions, and on determining what public health interventions can be effective and sustainable under real-life conditions. Currently, public health approaches for periodontal disease prevention and control are lacking. This review traces the historical strategies for prevention of periodontal disease in an epidemiologic transition context, using a modified model developed for cardiovascular disease, and presents a possible public health approach. Improving periodontal disease prevention and control will need to take into consideration the core activities of a public health approach: assessment, policy development, and assurance (Janakiram and Dye 2020).

The development of a comprehensive public health strategy to prevent periodontal disease and improve oral health is still in a nascent stage. For action to reach the scale that has been seen for public health intervention on cardiovascular diseases, awareness of the impact on health associated with periodontal disease must be clearly articulated to frame periodontal disease as a public health problem (Enwonwu and Salako 2012).

One important way forward is the recognition of the value that public health informatics could add to a comprehensive strategy in areas of surveillance, prevention, and health promotion. Using the newer tools of data science and technology, public health informatics can help with transforming periodontal disease assessment that better informs decision making. This can facilitate public health policy that supports environments and living spaces that promote periodontal health, encourage oral health literacy leading to individual empowerment, and identify strategies that support integration of periodontal health care into overall health care.

II.2.2. MANAGEMENT OF PERIODONTAL DISEASE

The predictable regeneration of intrabony defects remains an important goal in the management of periodontitis. Clinical and histologic evidence of periodontal regeneration has been shown for multiple regenerative therapies, including bone replacement grafts, guided tissue regeneration, and biologics, when used alone or in combination. Regenerative therapies improve periodontal health, as evidenced by gains in clinical attachment level, reductions in probing depth, and gains in radiographic bone fill. Important patient-related factors (e.g., smoking) and defect/site-related factors (e.g., defect morphology and gingival biotype) can influence the potential to achieve periodontal regeneration. The regeneration of intrabony defects generally becomes more challenging with increasing loss of height, proximity, and number of bony walls.

Therefore, combination therapies may be necessary to achieve predictable regeneration. Clinical improvements after regenerative therapy can be maintained over extended periods (≥10 years) with professional maintenance at appropriate intervals and adequate home care (Reynolds et al., 2015).

Alveolar ridge augmentation either before or during implant placement is a predictable procedure under certain conditions. Also, periodontally accelerated osteogenic orthodontics, a newly evolved technique, involves a combination of corticotomy followed by placement of bone graft and orthodontic force application for the closure of the extraction space (Ibraheem et al., 2021).

Recent reports have stated that there is a relationship between dental malocclusion, psychosocial well-being, and self-esteem. In addition, the current trend is that we have more of adult patients seeking orthodontic treatment in order to improve their facial appearance. It is estimated that approximately 12–24 months is the time needed for comprehensive orthodontic treatment, which in turn depends upon severity, treatment plan, and individual characteristics (Wang et al., 2009). Prolonged treatment time can be an added risk factor for other dental problems such as root resorption, caries, and periodontal disease due to poor oral hygiene.

To reduce orthodontic treatment time due to patient demand, orthodontists have tried to accelerate tooth movement using various methods such as photobiomodulation, pharmacological approaches, and low-intensity laser irradiation. Among all these procedures, surgical procedure, i.e., periodontally accelerated osteogenic orthodontics technique (PAOO), has widely been popularized, as it significantly reduced orthodontic treatment time (Rugnami et al., 2018).

Regional accelerating phenomenon (RAP) is the main biological mechanism behind the acceleration of orthodontic tooth movement, which was proven by most of the animal studies. RAP has been defined as a re-organization activity and physiologic event that happened next to the site of injury, resulting in regional reduction in bone density in the healthy tissue (Thind et al., 2018).

The rationale behind this particular method comprises careful alveolar decortication, which is a form of periodontal tissue engineering causing transient osteopenia and high turnover adjacent to the injury site. Alveolar decortication initiates a healing response, the amount of which is directly related to the intensity and proximity of the surgical insult (Venkataramana et al., 2019).

Bone remodeling after tooth extraction and immediate implant placement will occur nonetheless and, as a result, additional hard and soft tissue augmentations are often necessary to compensate for the loss of alveolar ridge dimension. The socket shield (SS) technique has shown encouraging clinical results in maintaining original ridge morphology, and thus, may be used as an alternative protocol for the conventional immediate implant placement in the esthetic zone. The SS technique produces virtually no change in the hard and soft tissue dimensions with relatively minimal invasive surgical interventions and shorter treatment time (Nguyen et al., 2020).

The conventional socket shield (SS) design extends from the mesiolabial to the distolabial line angle. C-shaped SS, L-shaped SS, and proximal SS designs have proximal extensions that help maintain the hard and soft tissue in the interproximal areas. This is beneficial for implant sites adjacent to an existing implant or an edentulous space. The most common complication of the socket shield technique (SST) is internal shield exposure. Due to anatomical features such as a scalloped ridge shape and an oval socket shape of some teeth, the risk of complications such as internal shield exposure, inadvertent SS displacement, and fracture of the SS during implant insertion is greater in proximal shield areas (Pohl et al., 2022).

First reported in 2010, the SS technique had progressed from concepts introduced in the 1950s that the retention of a tooth limits tissue alterations following extraction. The submergence of tooth roots was introduced originally to preserve alveolar ridge volume beneath removable full prostheses. Malmgren and coworkers had also more than 3 decades ago reported successful tissue regeneration around submerged tooth roots. Thereafter, submerging a tooth root for pontic site development has become a well-documented treatment (Bäumer et al., 2015). Salama and co-workers reported on preserving the entirety of the attachment apparatus as well as on the complete preservation of the alveolar ridge when developing pontic sites beneath FPD. This technique typically decoronates the tooth at the bone crest or preferably mm above it so as to preserve the supracrestal fibers with epithelial and connective tissue attachment. By comparison, ridge preservation techniques may reduce the amount of ridge resorption but cannot prevent the loss of interdental bone and papillae (Gluckman et al., 2015).

Preservation of supracrestal fibers however can better develop pontic sites by in turn preserving the papillae. And thus, it has been shown that the retention of part of the tooth contiguous with the PDL, its fibers and reticulate vascularity interconnected with bundle bone, eludes the physiological remodeling of an extraction socket and the alveolar crest. These delicate tissues can be preserved – PDL, bundle bone, buccofacial plate, and overlying keratinized mucosa (Salama et al., 2007).

It can be postulated that retention of part of the tooth as a SS eludes the body from realizing the tooth has been extracted and circumvents the normal events of physiological healing that would resorb the alveolar socket. The resorption of a post-extraction socket is the direct result of trauma to the bone-PDL-tooth complex. Bundle bone born from a functionally loaded PDL is lost following extraction and sees an almost certain recession of residual buccofacial tissues (Filippi et al., 2001).

Complete maintenance of ridge volume after tooth extraction with preservation techniques utilizing currently available materials as a primary prevention is not yet possible. However, as stated before, the retention of tooth roots in the alveolar process can preserve the ridge tissues. Histologically, this was demonstrated by Hürzeler and co-workers. Their report confirmed the retained attachment of the SS to the buccal plate via a physiologic PDL free of any inflammatory response. The buccal plate crest showed an absence of osteoclastic activity – an absence of active remodeling (Hürzeler et al., 2010).

III.2.3. PREDICTIVE MANAGEMENT AND BIOMOLECULAR/BIOACTIVE COATINGS IN DENTAL IMPLANTS

Various statistical methods can be used for risk factor assessment; dental implant research predominantly uses logistic regression models with stepwise selection of risk factors. However, stepwise methods need to be adapted to dependent outcome data in cohorts where patients have varying numbers of implants. Moreover, stepwise methods often underperform in low-EPV patient cohorts. The creation and standardization of a protocol for predictive evaluation of post-implant dental evolution represents one of the main goals of my career and a major research direction.

Implant osseointegration is a prerequisite for clinical success in orthopaedic and dental applications, many of which are restricted by loosening. Biomaterial surface modification approaches, including calcium-phosphate ceramic coatings and macro/microporosity, have had limited success in promoting integration. Regarding biomolecular coatings, which have been recently developed and studied, good results were observed in animal experiments. However, reproducing actual cells and tissues is not easy because of the involvement of several variables. External environmental changes such as different pH levels, the presence of oxidants, and ultraviolet exposure can impair cell function or trigger cell death owing to the limitations of the cells. In addition, transplanted therapeutic cells die easily owing to inflammatory and immune responses. Efforts to overcome this problem through multilayer cell coating can provide avenues for diagnosis and basic cell biology studies owing to the following features of multilayer films: high capacity available for attaching different biomolecules; natural replication of signal molecule diffusion across cells; and the possibility of cell patterning. Furthermore, light-triggered release from multilayer films achieves the delivery of biomolecules with a high spatiotemporal resolution (Choi et al., 2020).

Metallic implants sometimes fail in surgeries due to inadequate biocompatibility, faster degradation rate (Mg-based alloys), inflammatory response, infections, inertness (SS, Ti, and Co-Cr alloys), lower corrosion resistance, elastic modulus mismatch, excessive wear, and shielding stress. To improve osseointegration, titanium surfaces were coated with the glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine (GFOGER) collagen-mimetic peptide, selectively promoting alpha2beta1 integrin binding, a crucial event for osteoblastic differentiation. Titanium surfaces presenting GFOGER triggered osteoblastic differentiation and mineral deposition in bone marrow stromal cells, leading to enhanced osteoblastic function compared to unmodified titanium.

One the major issues after dental implant is peri-implantitis. Surgical procedures for peri-implantitis treatment include two main approaches: non-augmentative and augmentative therapy. Open flap debridement (OFD) and resective treatment are non-augmentative techniques that are indicated in the presence of horizontal bone loss in aesthetically nondemanding areas. Implantoplasty performed adjunctively at supracrestally and buccally exposed rough implant surfaces has been shown to efficiently attenuate soft tissue inflammation compared to control sites. However, this was followed by more pronounced soft tissue recession. Adjunctive

augmentative measures are recommended at peri-implantitis sites exhibiting intrabony defects with a minimum depth of 3 mm and in the presence of keratinized mucosa. In more advanced cases with combined defect configurations, a combination of augmentative therapy and implantoplasty at exposed rough implant surfaces beyond the bony envelope is feasible (Ramanauskaite et al., 2020).

For a long-term application of bioimplants, surface characteristics and their biological functions are considered as key factors. Engineering the surface of the biomaterials by applying suitable coatings provides flexibility in tailoring the properties as per the requirements (Wijesundara et al., 2023) .

Bioceramic coatings hold great potential by tailoring the biological properties that suit our needs: the choice of the coating depends on the interaction between the cells with the coatings and substrates that are being used. Coatings on metallic implants are invaluable due to their functionality, biocompatibility, durability, and stability. Bioactive coatings are used to enhance the biological fixation between the bone and metallic implant despite their poor tribological and mechanical properties. Hence, they are often improved by developing composites with materials that possesses good mechanical strength.

II.3. FUTURE DIRECTIONS IN TEACHING ACTIVITY

I believed that the key to career success was making regular efforts to expand one's knowledge base by reading, study, and attendance at scientific conferences, both locally and abroad. As soon as I made contact with the newcomer in the area, I set out to learn the necessary technical abilities to include it into my daily work and my theoretical and practical teaching.

Students entering the field of Periodontology come to the course with specific assumptions about the nature of their clinical work and their interactions with patients. A proper educational act relies on a number of pillars, one of the most crucial being an adequate environment in which to carry out practical work. Moreover, in fields with a heavy emphasis on practical application, like periodontology, it is crucial that all students have experience carrying out therapeutic procedures, first on a simulator and then on actual patients when they are ready.

A lot of pressure may be put on the faculty since a teacher's influence on a student's growth can be either positive or negative. As students have access to more and more forms of knowledge, their backgrounds, experiences, and expectations all shift throughout time. Keeping up with their ever-evolving preferences is essential. During the current COVID epidemic, educational institutions face unprecedented challenges. Our students have just realised that classical pedagogical practises, which they had written off as irrelevant, have actual significance and should be reinstated among the tools used in medical education. For us educators, this means striking a balance between time-tested practises and cutting-edge tools like computer-assisted instruction.

Within academic fields, lecturing plays an integral role in creating the next generation of academics and professionals, granting the skills, abilities and knowledge to get them started on their chosen career. But continually improving as a lecturer is a challenge in itself.

The first step to improving as a lecturer is to understand the kind of qualities that make for a good lecturer. These can include things like:

- **Being engaging** – Having a lecturer who is switched on, always ready to spark discussion and communication, and always open to finding new ways to engage students is key to keeping things fresh and interesting.
- **Being compassionate** – All students are human beings with their own personal lives. Being understanding when things happen in these personal lives is essential for supporting your students to be the best learners they can be.
- **Being enthusiastic** – Enthusiasm as a lecturer will keep you interested in everything you have to teach, and that passion for your subject will come through in your teaching and higher education skills, gifting your students with an enthusiasm for learning.
- **Being proactive** – Taking an active interest in your students will make them feel valued as part of the education institution. Check in with your students regularly to ensure your teaching is doing its job and that all class members are able to keep up.
- **Being approachable** – Having a lecturer who's hard to approach as a student can quickly derail progress on a module. Try to ensure you're as welcoming to everyone in your college or university as you can be to ensure a positive lecturer-student academic relationship.

A teaching figure's influence on a young practitioner's growth can be beneficial or detrimental, which can place a lot of strain on the teaching staff. As exposure to different sorts of knowledge develops, both the student profile and their expectations are always changing. This necessitates ongoing adjustments to meet their shifting demands..

►**Future directions for teaching activities for students include:**

- ✓ diversifying instructional-educational methods;
- ✓ using some instructional-educational methods that tap into students' creative potential;
- ✓ tailoring and differentiating course curricula to suit the needs of each educational context;
- ✓ increasing the use of information technology in the teaching activity;
- ✓ participating in international mobility programmes like exchange programmes.
- ✓ presentation must be backed up by sound reasoning to help students learn to think medically and develop both their broad and deep intellectual skills in preparation for a career in dentistry.
- ✓ student engagement in the teaching and learning process, the resolution of fictitious clinical scenarios, simulations, virtual patients, research projects, etc., are all encouraged both during and after the traineeship.
- ✓ preparing the students to cope with professional stress
- ✓ creation of unbiased methods for gauging students' engagement and progress in the classroom,

- ✓ assessment criteria are developed in accordance with the learning outcomes, guaranteeing a fair and accurate assessment of student performance.
- ✓ Increasing the scope of available education by using cutting-edge methods of instruction including AI, AR/VR, and teledentistry
- ✓ activities that help students build the crucial interpersonal communication skills needed to work effectively with patients and with one another
- ✓ cooperation with other national centres to promote student mobility and increase student involvement in exchange programmes across educational institutions.
- ✓ meeting with students to discuss academic concerns, career advice, social support, etc.

►**Future directions for post graduate students:**

- ✓ teaching activities providing current information through the use of databases, websites, and specialised publications;
- ✓ conducting post-academic refresher courses tailored to the Periodontology residency curriculum;
- ✓ involving residents in the planning of public health education campaigns;
- ✓ and participating in global mobility initiatives like the Erasmus+ programme.

The clinical traineeship we provide our students allows us to get to know them on a more personal level, to learn more about their strengths and weaknesses, and to tailor our instruction and assessment accordingly.

►**Future directions for PhD students**

The job of a Phd thesis supervisor entails a number of commitments made at the moment the PhD applicant accepted my offer to organise, oversee, and effectively complete their work. Since the PhD thesis supervisor's role is crucial to the execution of a fruitful research project, I would like to state that I am completely aware of it. PhD candidates' activities are directed towards:

- ✓ selecting the most qualified students interested in enrolling in the doctoral programme;
- ✓ assisting PhD candidates in: writing the research study protocol;
- ✓ selecting the appropriate scientific methods needed to carry out the research; forming collaborations with experts in the fields of medical biostatistics and epidemiology, as well as researchers from prestigious national institutions;
- ✓ supporting PhD candidate mobility via training and promoting participation in sessions for the dissemination of scientific findings throughout the academic community.

I understand that coordinating PhD applicants is a lot of extra work on top of what I'm already doing in academia, but I'm certain that my background in teaching and research will help me succeed in this role.

►Other activities relating to professional development

In addition to my studies, one of my main concerns will be educational projects. In order to advance my career prospects, I plan to join an international research team and discuss the findings of my studies at prestigious conferences dedicated to oral public health.

The ultimate goal of our never-ending quest for knowledge should be to put it to use and profit from it. The acquired knowledge makes it possible to modify and enhance some approaches and procedures in the fields of education and dentistry practise, as well as to develop and introduce new ones.

II.4. CORRELATION OF RESEARCH, EDUCATIONAL AND MEDICAL ACTIVITIES

I plan to maintain a healthy work-life balance while I continue to hone my abilities across the academic, professional, and scientific spectrums in the years to come. To keep up with the ever-expanding body of medical knowledge, I plan to continue my education both independently and through formal participation in national and international scientific meetings and conferences hosted by eminent dental organisations like the European Federation of Periodontology (EFP), the Romanian Society.

As a member of the Romanian Society of Periodontology, I want to actively pursue opportunities for academic exchange with my colleagues both inside Romania and beyond. I plan to direct my attention on creating and studying the following areas of inquiry:

►Maintain and complete the studies of individuals with periodontal disease to determine their dental health and any correlations to systemic disorders;

►Results will be published in publications indexed by worldwide scientific indexing services;

►Together with other medical specialties (cardiology, nephrology, diabetology, oncology, gastroenterology, etc.), addressing new proposals that could provide real funding through joint research projects;

►Maintaining and expanding collaboration with preclinical disciplines like, Microbiology, Immunology, Biochemistry, Medical Genetics, Pharmacology and other dental disciplines (prevention, cariology, endodontics, pedodontics, orthodontics, prosthetics);

►Attracting funds to improve Periodontology research means;

►Expanding collaboration with other research laboratories outside the faculty; organising and participating in national and international scientific events; expanding periodontology research through participation in national grants;

►Developing applications for international research programmes and grants;

Concerning innovations, I will make an effort to keep up with the newest findings in periodontology in order to revise my diagnostic and treatment methods so that they are in line

with international standards. I plan to train harder in areas where I feel I am weakest so that I can broaden my skillset.

Evolution's results are not limited to adaptation; cooperation and coevolution are also possible consequences. For mutual success and growth, periodontists and other dental and medical professionals must work together and support one another.

The majority of professional categories are required to advance in just one area. We belong to the group of categories that simultaneously needs development on a number of fronts. This increases the value of achieving extraordinary achievements.

In an effort to strengthen each direction through the other two, I will strive to approach the three directions of activity in a productive and balanced manner.

I'm thinking about the following things:

- ✓ improvement of current courses in light of current potential and needs the inclusion of virtual patients in the curriculum; the promotion of periodontology innovations;
- ✓ the application of research findings in clinical practise; or the active involvement of students in the research, educational, and medical educational processes;
- ✓ the coordination of diploma papers; and the coordination of research topics within student scientific circles.

II.5. CONCLUSIONS

In conclusion, persistence, an open mind to novel ideas, the ability to communicate within work teams, and the continuous development of teaching and professional performances are the keys to the success of the academic profession. I believe that the department, faculty, and university where I presently work will become more well-known as a result of my professional reputation and potential academic career.

I am pursuing both a continuous process of professional and personal growth as well as a contribution to the training of future generations of experts towards high human and professional quality through the objectives for the development of the university career that are provided in this plan.

SECTION III
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