GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

HABILITATION THESIS

CLINICO-BIOLOGICAL RESEARCH REGARDING PERIODONTAL MODIFICATIONS ASSOCIATED TO SYSTEMIC DISORDERS IN YOUNG POPULATIONS

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HABILITATION THESIS

ABSTRACT

Teaching can be challenging at times, and need to care about students and have a strong motivation to perform classes and understanding with them, both, from the provided materials and their struggles outside the class point of view being also very rewarding. Teaching is therefore one of the most fruitful professions in that it gives an opportunity to make an impact on a future generation. It is also extremely difficult and draining, being a teacher involving patience, dedication, passion, and the ability to do more with less. It is a treacherous journey often filled with just as many valleys as there are mountains.

The habilitation thesis, which sumarizes my postdoctoral professional, academic and scientific activity, is framed in three major sections, according to the CNATDCU recommendations. The paper entitled *Clinico-biological researches regarding periodontal modifications associated to systemic disorders in young populations* reflects the overview of my concerns in the fields of oral status modifications in the young population associating several general disorders, bothe form the clinical and preclinical point of view, based mainly upon the experience achieved during the doctoral training program and continued afterwards.

The first section relies upon some personal, professional, academic, scientific achievements and a summary of my PhD thesis. My PhD written dissertation entitled ***Changes in the composition of crevicular fluid in periodontal disease in children and adolescents*** and defended in 2004 was dedicated to the study of various risk factors for the periodontal impairment in children and teenagers associating several immune-inflammatory and genetic disorders, which often raise special issues in establishing the diagnosis and elucidating the mechanisms of the disease as well.

Particularly, the main subjects of my scientific interest have targeted two main directions: ****clinical studies reflecting*** the emerging concepts regarding the occurrence and development of periodontitis in children and adolescents and ****laboratory findings*** in order to assess either binomial relationship between periodontal impairment and various systemic conditions in young populations, sheding an important light upon some biochemical and microbiological issues that impact systemic homeostasis.

During this part of the thesis, there is an overview of the scientific research grants which have been either managed or been involved as a team member, revealing the data related to the aim and activities carried out, and some of the results obtained in the research I have conducted as a project director. Moreover, in this first part there are some essential elements that could highlight the national and international visibility, with particular reference to the citations of papers published in indexed ISI Thomson Reuters and in international databased recognized journals.

The second section spots the scientific achievements reunited during the 14 years that followed the doctoral research, emphasizing the three main directions of the research:

1. Analysis of the periodontal modifications in juvenile and adult population with associated systemic disorders
2. Gingival fluid – smooth interface in the assessment of the periodontal status
3. Laboratory investigations for assessment of general and oral homeostasis – a reappraisal from biochemical and pharmacological viewpoint

Considering these aspects, during my research work I have seeked for the investigation of the current concepts regarding the occurrence and development of periodontal breakdown in children and adolescents, making complex investigations. The bivalent relationship between diabetes and periodontal disease has been illustrated by Löe (1996) - "periodontal disease is the sixth complication of diabetes" and Grossi (1994) - "diabetes is the third risk factor (by age and smoking) of periodontal disease" since many years. Attempts have been made to highlight the role of risk factors in periodontal disease in healthy and affected groups of children and teenagers (via insulin-dependent diabetes mellitus and Down syndrome), our study using a quantification of inflammatory response that might impact the periodontal condition, resulting thus in significant clinical and statistical interpretations of the effects of systemic risk factor (insulin-dependent diabetes / Down syndrome) on periodontitis.

Since epidemiological data on the prevalence of periodontal disease in children and adolescents in Romania are extremely low, contrasting with those existing in relation to the prevalence of dental caries in this population, all the clinical, statistical, and laboratory investigations were conducted in the attempt to make a modest contribution to a better and deeper understanding of the pathological phenomena underpinning the onset and maintenance of periodontal disease in children and adolescents in different situations and to establish a proper diagnosis as early and closer possible to the state of the periodontal examination.

The third part of the habilitation thesis mirror the major directions for the intended future academic activities and researches, focused on development of: scientific projects proposals in the national and international competitions, optimization of the collection and analysis of the oral fluids and their impact in the relationships between periodontal impairment and systemic immune-inflammatory and metabolic disorders in children and teenagers.

The last section includes a list with the main references that have been consulted in order to sustain the data presented.

Rezumat

Procesul de predare reprezintă o adevărată provocare în activitatea curentă a unui dascăl, necesitând o grijă deosebită față de studenți și o motivație în susținerea cursurilor și prelegerilor astfel încât să existe o empatie atât din punct de vedere al materialelor oferite, cât și din punctul de vedere al dificultăților pe care aceștia le întâmpină în afara orelor de curs. Acelașii proces oferă însă și foarte multe satisfacții. Astfel, meseria de dascăl este una din cele mai emblematice, oferind oportunitatea de a-ți pune amprenta asupra generațiilor viitoare. Este, de asemenea dificilă, epuizantă, impune răbdare, dedicație, pasiune și abilitatea de a face multe din puțin. Este o călătorie adesea plină de capcane, cu la fel de multe suișuri ca și coborâșuri.

Teza de abilitare, ce rezumă activitatea mea post-doctorală, științifică și academică, cuprinde trei secțiuni majore, conform recomandărilor CNATDCU. Lucrarea intitulată *"Cercetări clinico-biologice ale modificărilor parodontale asociate afecțiunilor sistemice la populațiile tinere"* reflectă preocupările de ansamblu în ce privește modificări ale homeostaziei orale în populațiile tinere cu afecțiuni generale severe, atât din punct de vedere clinic, cât și paraclinic, în principal pe baza experienței acumulate în timpul programului de pregătire doctorală și continuată ulterior.

Prima secțiune este construită pe baza unor realizări personale, profesionale, academice și științifice și include un rezumat al tezei mele de doctorat. Disertația scrisă a doctoratului, intitulată ***Modificări în compoziția fluidului crevicular în boala parodontală la copii și adolescenți*** susținută în 2004, a fost dedicată studiului unor variați factori de risc ai dereglarii homeostaziei parodontale la copiii și adolescenții cu afecțiuni imuno-inflamatorii și genetice severe, ce impun adesea cerințe speciale atât în stabilirea diagnosticului, cât și în elucidarea mecanismelor bolii.

Subiectele principale ale cercetării științifice au urmărit două mari direcții: ***studiile clinice** vizând conceptele inovatoare referitoare la apariția și dezvoltarea bolii parodontale la copii și adolescenți și ***investigații de laborator** pentru a evalua relația binominală dintre implicarea parodontală și variate condiții sistemice la populația tânără, aducând completări și idei noi asupra unor aspecte biochimice și imunologice care modifică homeostazia locală în context sistemic.

Materialul descris în teză cuprinde și un sumar al granturilor de cercetare științifică la care am participat în calitate membru, expunând datele legate de scopul și activitățile întreprinse, precum și unele din rezultatele obținute în cercetările pe care le-am condus ca director de proiect. Mai mult, în această secțiune sunt prezentate repere importante ce susțin vizibilitatea națională și internațională, cu referire în special la citări ale lucrărilor publicate în reviste indexate ISI Thomson Reuters și în jurnale recunoscute internațional.

A doua secțiune notează realizările științifice de pe parcursul celor 14 ani ce au urmat cercetării doctorale, cu accent pe cele trei mari direcții de cercetare:

- Analiza modificărilor parodontale la populația juvenilă și adultă cu afecțiuni sistemice asociate
- Fluidul gingival – interfață utilă în evaluarea statusului parodontal

- Cercetări de laborator pentru evaluarea homeostaziei generale și orale – o reevaluare din punct de vedere biochimic și farmacologic

Luând în considerare aceste aspecte, de-a lungul cercetării mele am căutat să investighez conceptele curente referitoare la apariția și dezvoltarea afecțiunilor parodontale la copii și adolescenți, realizând investigații complexe, la nivel analitic și clinic. Relația bivalentă dintre diabet și boala parodontală a fost ilustrată cu mulți ani în urmă, de Löe (1996) – ”boala parodontală este a șasea complicație a diabetului” și Grossi (1994) – ”diabetul este al treilea factor de risc (după vârstă și fumat) al bolii parodontale”.

S-a încercat sublinierea rolului factorilor de risc în boala parodontală la grupuri de copii și adolescenți sănătoși și bolnavi (cu diabet insulino-dependent și sindrom Down), studiul nostru utilizând o cuantificare a răspunsului inflamator ce ar putea afecta statusul parodontal, rezultând astfel interpretări clinice și statistice semnificative ale efectului factorilor de risc sistemici (diabet insulino-dependent / sindrom Down) asupra alterărilor din teritoriile parodontale.

Datorită paucității datelor epidemiologice asupra prevalenței bolii parodontale la copiii și adolescenții din România, cele existente fiind legate strict de prevalența cariilor în această populație, toate investigațiile clinice, statistice și de laborator s-au realizat în încercarea de a aduce o contribuție modestă la o mai bună înțelegere a fenomenelor patologice subjacente apariției și dezvoltării bolii parodontale la copii și adolescenți în diferite situații și pentru a stabili un diagnostic potrivit, cât mai precoce și cât mai corect, al dezechilibrului la nivel oral în general și parodontal, în particular.

A treia parte a tezei de abilitare oglindește direcțiile majore ale viitoarelor activități didactice și de cercetare, țintite către dezvoltarea de proiecte științifice viitoare, în competiții naționale și internaționale, optimizarea colectării și analizei fluidelor orale și rolului lor în relația dintre boala parodontală și afecțiunile sistemice imuno-inflamatorii și metabolice la copii și adolescenți.

Ultima secțiune include o listă a principalelor lucrări de referință consultate în vederea susținerii datelor prezentate.

SECTION I

OVERVIEW OF PERSONAL, PROFESSIONAL, ACADEMIC AND SCIENTIFIC ACHIEVEMENTS

One of the most challenging aspects of being a teacher is that we must be able to see the forest and the trees at the same time. Hopeless visionaries who cannot manage the daily details of teaching do not last too long, but if we become unimaginative micromanagers, we quickly lose sight of the greater goals and aspirations that fuel our fire and drive us to be grgood instructors.

One of our fundamental human needs is to have a sense of significance. We need to know that our work is important and that we are having a positive influence on others, and this impact is one of the great strengths of our profession. These represent the didactic and scientific foundation of my university progress, which began more than 20 years ago. The career development plan follows: the professional and didactic activity: highlight of the earned experience, achievements and future plans; the research activity, either from the former activity, scientific results and future goals.

I.1. Professional progress

The beginning of my professional career is tightly related to the graduation the Faculty of Dentistry, University of Medicine and Pharmacy "Grigore T. Popa" Iasi, in 1995. Since than, I activated in primary dental assistance, being a specialist doctor - *Dental Practitioner* since 2000 (confirmed by the Degree issued by the Ministry of Health 1011/ 2000 – Diploma Series, no. S1, no. 002228, no. DB 2230/01.06.2009). In 2005 I became senior specialist in General Dentistry (confirmed by the Degree issued by the Ministry of Health 971/ 2005 – Diploma Series, no. P1, no. 000103, no. DB 970/04.05.2009) and in 2008 I completed already the second specialty in "*Orthodontics and dental-facial orthopedics*", becoming junior specialist in Orthodontics and dental-facial orthopedics after validating another exam (the Degree issued by the Ministry of Health 2025/ 2008 – Diploma Series S1, no. 001567, no. DB 1523/31.03.2009). Finally, in 2017 I've been confirmed as Junior Specialist (confirmed by the Degree issued by the Ministry of Health 492/ 02.05.2017 – Diploma Series, no. S1, no. 033606, no. DB 32557/12.06.2017, and, afterwards, Senior specialist, in *Pedodontics* (confirmed by the Degree issued by the Ministry of Health 510/ 02.05.2017 – Diploma Series, no. P1, no. 019134, no. DB 17093/14.02.2019).

In all these years, I continued developing my professional education, by involving in post-graduate training courses, scientific and research-related projects. I have participated at various international and national courses with international participation, national courses and conferences, all related to topics that involve the young subjects and their oral and general health status, considering the clinical view in tight connection with

the possible preclinical investigations (immunological, biochemical and morphological determinations).

I.2. Academic activity

Main didactic position acquired through contests:

- Junior assistant at - "Sf. Apollonia" University of Iasi (tarting with. 01.10 1996 since 01.12.1998)
- Assistant professor – in the Pediatrics Department of the Faculty of Dental Medicine, University of Medicine and Pharmacy "Gr. T. Popa" Iasi, (2001untill 2009)
- Lecturer – since February 2009 untill October 2015;
- Associate professor – since October 2015 until present, at the Surgical Department, Faculty of Dental Medicine, 'Grigore T. Popa' University of Medicine and Pharmacy, Iași.
- In 2005 I became a PhD in Dental Medicine - Confirmed by Order MEC no. 3956 / 25.04.2005.

Over time, the preparation for the teaching career have been developed in parallel with the specialized scientific research activity.

Considering my teaching commitment in the Dental Medicine Faculty, I developed various activities, according to my academic degree, as follows:

- Junior assistant – University of Dental Medicine "St. Apollonia "Iaşi
  - clinical training in Dental Prevention Year III of study
  - clinical training Parodontology year VI of study
- Assistant Professor - Faculty of Dental Medicine, University of Medicine and Pharmacy "Gr. T. Popa" Iasi
  - clinical training - Pedodontics Year III, IV, VI - Dental Medicine Faculty
  - clinical/laboratory training - 2nd year College of Dental Technicians
- Lecturer - Faculty of Dental Medicine "UMF Gr. T. Popa" Iasi
  - teaching courses and clinical training in Pedodontics Year III, IV, VI
- Associate Professor at the Faculty of Dental Medicine, University of Medicine and Pharmacy "Gr. T. Popa" Iasi
  - Teaching courses and clinical training - Pedodontics Year III, IV, VI of study
  - Teaching in Pedodontics, lines of study in Romanian and French language

In all these years, I have also been involved as a lecturer or coordinator in several optional courses for Pedodontics for the 4th and 6th year students for Romanian programs and also for students enrolled for College of Dental Technicians of the 2nd and 3rd years.

For these courses, I designed modern presentation materials (power point and video materials for indicating the particularities on the dental and periodontal status upon young children affected by various general conditions, the clinical manifestation and their

reflection at the paraclinical level, using protocols to assess the active state of the disease). These courses have issued materials that were further uploaded online on the e-learning platform available at the 'Grigore T. Popa' University of Medicine and Pharmacy in Iasi. The lectures were individually adapted for medical assistance and dental technicians with detailed aspects of clinical and preclinical grounds, for each specialization, making use of the graphic and video materials specific to the course topic.

During the practical classes, I have been focusing on the practical experience of the technicians by illustrating the importance of prevention which include instruction in the proper diet, use of fluoride and practice of oral hygiene.

The teaching profession offers the privilege and the challenge to activate in three main directions:

- Education
- Health
- Research

Each of these areas requires continuous training to meet specific, ever-increasing requirements, while a solid career under these conditions implies maintaining standards and achieving performance that equally targets all three plans.

The university career in dental medicine is one of the means to accomplish a successful career in clinical practice, while offering the opportunity to contribute to the formation of new generations of dentists and to remain anchored to current trends through research.

As teachers we also have the role of: cultivating students' passion for the chosen profession and creating the patients, in our case children, the emotional comfort needed to conduct dental therapies; acquiring, through continuous training, of new knowledge, skills and competences in the three areas: education, research and dental medicine; obtaining solid knowledge in related fields of dentistry with a view to interdisciplinary approach to dental problems.

At the same time, my academic activity has been duplicated and multiplicated, initially based mostly on seminars, with my advance to lecturer and, afterwards, as Associate Professor, a position that has been an important step in choosing new directions and sub-fields of work that are still insufficient explored, in view of the specificity of the activity in the faculty of dentistry. Therefore, starting with 2009 I was part of various research groups as a member of the research projects focusing on the immunoinflammatory response during periodontitis associated to general disorders such as metabolic imbalances or genetic disorders.

As an academic, I have been part of committees responsible with the organization of eight scientific international conferences, as well as member of the committees for organizing and supervising national scientific programs, especialy those that already became tradition, the WHO (World Health Organization) Day, for several editions.

I.3. Scientific research activity

I have been admitted to be PhD training at the 'Grigore T. Popa' University of Medicine and Pharmacy Iaşi, Faculty of Dental Medicine in 2000 and in 2005 I was awarded the title of Doctor in Medical Sciences, Pedodoncy specialty (Doctor's Degree Series D no. 0002869, Order of the Minister of Education and Research no. 3956 / 25.04.2005) – with the thesis entitled: "Changes in Crevice Fluid Composition in Marginal Periodontal Diseases in Child and Adolescent", Prof. Dr. Maxim Adam.

During the preparation of the PhD thesis I updated and improved a series of experimental techniques and models of experimentaly-induced diabetes for laboratory animals. My doctoral thesis has been divided into two main parts: current knowledge and personal contributions. The general part of the thesis contains theoretical aspects concerning the particularities of the marginal periodontium in children and teenagers, the puberal changes and the possible algorithms for using gingival crevicular fluid as an interface between the dental and systemic status.

After obtaining my PhD in Medical Sciences, I have strengthened my research on diabetes in children and adolescents, oral manifestations of Down's syndrome, and changes in gingival fluid parameters in gingival inflammation, as well as in other directions.

The concepts of the pathogenesis of marginal periodontitis have undergone numerous changes as fundamental disciplines have brought new data that allowed a deeper understanding of the mechanisms triggered by incriminated etiological factors and as the processes taking place in the intimacy of tissues were better known. The prevalence, onset, progression, and especially the pathogenesis of periodontal disease can be modified by many endogenous factors, notably the fact that 50% of the risk of developing periodontal disease later in life is genetically determined and therefore present during childhood. Risk factors represent the category of factors that have been shown to be significantly associated with the increase in the prevalence of certain specific diseases.

The inconsistency between the aggressiveness of periodontal destruction in children and adolescents and the amount of bacterial plaque in some forms of periodontal disease has led a number of researchers to say that bacteria, although absolutely necessary for the development of periodontal disease, are insufficient (as conditions) for to develop periodontitis

Therefore, my research directions have been focusing upon:

- analysis of the periodontal modifications in juvenile and adult population with associated systemic disorders;
- gingival fluid – smooth interface in the evaluation of the periodontal status;
- laboratory investigations for assessment of general and oral homeostasis – a reappraisal from biochemical and pharmacological viewpoint.

The development of pathophysiology and immunology has brought tremendous data into understanding the stages of inflammation in the periodontal tissues and the way each organism reacts differently to bacterial aggression with genetically determined characteristics. Therefore, the possible mechanisms through which a series of systemic diseases (endocrine disorders, blood diseases, diabetes) create favorable conditions for the

development of aggressive forms of manifestation of periodontal disease, with extremely rapid evolution, have been evaluated.

Working in the field of Pedodontics I became aware that the anatomic-functional features of the periodontium in the child and adolescent, the variety of clinical expression of the disease, as well as the heterogeneity of the etiology and the complexity of the pathogenetic mechanisms, make periodontal disease in the child and adolescent still a subject with many unknowns for us, the clinicians. As a consequence, during my research activity, the first step targeted the study of the influences and effects of diabetes on the periodontal structures in children and adolescents; the **main goal**: *identification of early markers for the detection of periodontal alterations in young subjects with insulin dependent diabetes and thus significant in preventing the progression to severe destruction in oral territories with loss of periodontal support or dental units*.

Furthermore, the **specific purposes** envisaged implementation of dental sanogenic education programs on the primary prevention of dental caries and periodontal diseases as well as the interception of dentomaxial anomalies in pre-school and school age children

My experience in the area has begun since the students' participation in scientific events with papers in the field of oral prevention, finalized by the diploma thesis "Fluid ion transfer mechanisms of various fluorinated fluoride products *in vitro*", the participation at the Congress of Young Doctors (October 1997, Berlin, Germany) with the theme "Enamel fluoride *in vitro* transfer", the guidance of some diploma papers on this topic, as well as participation in numerous actions consisting of dental hygienic sanitary education lessons in kindergartens, schools (Carol, Ion Creangă, Leţcani), high schools (Economic, Theological). These actions resulted in: partnership projects ("Ion Creangă" School), contests (member of the jury of the "Little Sanitaries" County Competition, 2012, within the partnership between ISJ – School Inspectorate Iaşi and the National Red Cross Society in Romania - Iasi Branch) , workshops that included the topic of sanitary education, as well as participation in the community actions: "Moldavian prophylaxis days" (2000), "The magic brush" (2013), "Special Smiles" component of Special Olympics - Sports Healthy (2012).

The purpose of the study is to educate children and adolescents regarding their oral health, their parents and educators, to raise awareness of the importance and benefits of preventing dental caries, gingivitis and dentomaxial abnormalities.

The results achieved by the study of the immuneinflammatory response during periodontal-systemic disease association may represent the important starting points for the investigations' extension on other biochemical and microbiological level, in order to clarify certain aspects related to the activity variation depending on the severity of periodontal impairment, which could provide real benefits to the development of new biological markers of the disease activity status.

Reuniting specialists form many areas, both medical and non-medical fields (such as chemists and informaticians, practitioners in microbiology and biochemistry) have made possible these projects to be interdisciplinary, in order to perform complex fundamental investigations according to the European requirements.

Some of the results obtained during the researches performed were published in ISI Web of Sciences-indexed journals (**Toma V**, Cioloca DP, Forna DA, Hurjui L, Botnariu G, Nechifor IE, Bogdan M, Costuleanu M, Simion L, Holban C. IL-18 as an important gingival inflammatory biochemical marker in children and adolescents with insulin-dependent diabetes mellitus. *Rev Chim*. 2016; 67(12):2545-2551; Cioloca DP, Foia L, Holban C, Trandafirescu M, Poroch V, Maxim D, Jipu R, Costuleanu M, **Toma V**. Systemic diabetic context-induced biochemical periodontal alterations in children. *Rev Chim*. 2016; 67(12): 2409-2412; Rauten AM, Silosi I, Stratul SI, Foia L, Carmen A, **Toma V**, Cioloca D, Surlin V, Surlin P, Bogdan M. Expression of Pentraxin 3 and Thrombospondin 1 in Gingival Crevicular Fluid during Wound Healing after Gingivectomy in Postorthodontic Patients. *Journal of Immunology Research*, 2016: 1-7, 4072543) and in several journals recognized in the international databases.

The same preoccupations in the field made possible spreading the results by publication of one manuscript, and an international edited book chapter: "*Periodontal Pathology in Juvenile Diabetes*" (Book editor, Publishing House "Gr. T. Popa" UMF Iaşi 2008); *"Periodontal disease - a clinician's guide"* - Intech Ed, 2014, ISBN 978-953-307-818-2.

Over the years, the conducted studies on the evaluation of the oral and periodontal status in young subjects, further experimental researches has been conducted from the position of team member or project manager for grants such as: **Development of a genetic test to determine the predisposition of periodontal disease in children and adolescents with Diabetes Insulin-dependent Diabetes** (CNCSIS no 31GR 2007-2008, coordinator "Grigore T. Popa" University of Medicine and Pharmacy Iasi), **Poliagregated orthodontic therapeutic studies for the treatment of complex aspects of disorders in the maxillo-facial sphere (**CEEX Nr. 287/2006), carried out in the period 2006-2008, coordinator "Carol Davilla" University of Medicine and Pharmacy Bucharest, partner "Grigore T. Popa" University of Medicine and Pharmacy Iasi, **Multifunctional Platform for Optimizing Diagnostic and Decision Methods in Medical Services - PROMED**, (CEEX Nr. 3 / 05.10.2005), UMF "Grigore T. Popa" Iasi, carried out during 2005-2008, coordinator S.C. IPA SA Bucharest, partner of "Grigore T. Popa" University of Medicine and Pharmacy Iasi, **Regional Network of excellence in the field of micro-nano-biotechnologies and textile materials for medical applications - EUROTEXMED**, (CEEX Nr. 181/2006), coordinated by Gheorghe Asachi Technical University of Iasi, partner of "Grigore T. Popa" University of Medicine and Pharmacy Iasi.

Alltogether, these projects (and others) represented a significant part for integrating clinical and scientific research, preparing me for the next stage, the supervising of a new research programme as a grant manager: **"Optimization of diagnosis and status of periodontal disease activity in juvenile diabetic population by molecular and immunenzimatic analysis of gingival crevicular fluid"**, research contract no. 29237/2013, run between 2014-2015.

Other COST Actions such as CA15135 *"Multi-target paradigm for innovative drug identification in the drug discovery process (MuTaLig)"* or BM1104 "*Mass Spectrometry Imaging: New Tools for Healthcare Research*" enabled, from the statute of the Action

Management Committee, to participate at certain scientists meetings, with specific deliver of the obtained results and their further integration in the literature data.

I.4. Achievements in the scientific publication area

Book Author - 2 chapters in International treatises
Book Editor – 1;
Book chapter coauthor – 13;
Collaborator - 2 specialty books;
Articles published in extenso in ISI Web of Science Core Collection -indexed journals, with IF - 19
First / last / correspondent author in ISI listed papers - 15;
Coauthor in ISI listed papers - 4;
Coauthor in ISI quoted papers - 1;
First / last / correspondent author in international databases listed papers – 24;
Co-author in international databases listed papers – 21;
In extenso articles in ISI Proceedings at international scientific manifestations – 8
Oral presentations at international scientific meetings - 45
Poster presentations at international scientific manifestations - 10;
Citations in ISI Web of Knowledge- indexed journals - 102;

I.5. Recognition at the national and international level

I am currently member in four international scientific societies: The European Association of Pediatric Dentistry, European Federation of Periodontology, European Dental Education Association, and I activate through several national scientific societies such as: Society of Physicians and Naturalists Iasi, National Association of Pediatric Dentistry in Romania and Romanian Society fof Oral Rehabilitation.

Three *in extenso* published papers in ISI Web of Knowledge-indexed journals were awarded by ***UEFISCDI*** within the program "***Awarding of the scientific results***".

My scientific publications have already counted over 100 citations in ISI Thomson Reuters indexed journals.

SECTION II

PROFESSIONAL, ACADEMIC AND SCIENTIFIC ACHIEVEMENTS FROM THE POSTDOCTORAL PERIOD

II.1. ANALYSIS OF THE PERIODONTAL MODIFICATIONS IN JUVENILE AND ADULT POPULATION WITH ASSOCIATED SYSTEMIC DISORDERS

II.1.1. Immunoinflammatory response and underlying genetic contribution in the binomial relationship diabetes – periodontal disease

II.1.1.1 State of the art

The prevalence of type 1 (T1DM) and type 2 diabetes mellitus (T2DM), (predominantly T2DM) is predicted to increase by 54%, to over 54 million individuals by 2030 (Rowley et al., 2017)

Multiple published studies describe the reciprocal relationship between diabetes mellitus and periodontal disease. Moreover, rates of obesity are increasing in children, impacting the prevalence of type 2 diabetes mellitus and periodontal diseases (PD). The incidence of diabetes mellitus in children began increasing 20 years ago. Approximately 8.3% of the U.S. population is affected by the diabetic disorder, with nearly 19,000 cases of T2DM in children and adolescents (Wooton et al., 2018).

Between 2001 and 2009, researchers from the SEARCH for Diabetes in Youth study found that T2DM in children and adolescents ageing 10 to 19 years had increased to 21%. Because of this focus on improving oral health for children, primary care providers are integrating pediatric oral exams, including dental screenings, into child exams. Gingivitis or periodontal disease may be detected and prevented by performing early oral screenings during child medical visits (Centers for Disease Control and Prevention 2016).

A study of Boyd highlighted the importance of oral health in patients with diabetes. Patients diagnosed with T2DM are 2.6 to 4 times more likely to eventually develop periodontal disease and 15 times more likely to become edentulous than patients who are not affected by the mentioned metabolic disorder (Boyd et al., 2012). Hyperinsulinemia, the precursor to T2DM, changes the bacterial flora in the oral cavity, making the patient more prone to dental caries, gingivitis, periodontal disease, and tooth loss.

A two-way bidirectional relationship exists between the degree of hyperglycemia and the severity of periodontal disease, with diabetes increasing the risk of periodontitis, and periodontal inflammation negatively affecting glycemic control. The role of the practitioner is to understand the link between hyperglycemia and gingivitis in order to halt disease progression (Preshaw et al., 2012). It is well established that poor oral health can lead to adverse health outcomes; a higher incidence of periodontal disease in children with poorly controlled blood glucose levels resulting in negative health outcomes. Current

evidence suggests periodontal disease adversely affects health outcomes for children with T2DM (Borgnakke et al., 2013.).

According to Demmer et al., hyperglycemia contributes to the deterioration of periodontal tissue regardless of the etiology or type of diabetes mellitus (Demmer et al., 2012). Poorly controlled T2DM (elevated HbA1c levels) contributes to periodontal impairment. In contrast, well-controlled T2DM does not increase the incidence of periodontal disease.

Proinflammatory cytokines triggered by the inflammatory process in periodontal disease play a key role in regulatory responses, including disruption in the insulin level. When the cytokines are secreted inappropriately, it results in periodontal breakdown. This balance between proinflammatory and anti-inflammatory processes is crucial in the development of periodontal disease (Lopes et al., 2012).

By measuring glucose levels during routine dental probing, this population has an additional opportunity for early diagnosis and treatment of diabetes. This collaboration could revolutionize healthcare coordination, resulting in better health outcomes in multiple environments (Gaikwad et al., 2013).

Type 1 diabetes mellitus is a chronic metabolic disease of an autoimmune origin with early manifestation predominantly in the childhood. Its incidence has been rising in most European countries. Studies on differences in oral microflora or the impact of metabolic control of diabetes on periodontal health, indicate a higher risk of periodontitis in children with type 1 diabetes. In children with diabetes, the periodontal impairment usually manifests in the adolescence and sometimes even earlier. It was confirmed that there is an association between poorly controlled diabetes (higher HbA1c levels) and development of periodontitis, even in children with type 1 diabetes (Novotna et al., 2015).

It is probably environmental factors, that is, changes in the exposure to certain nongenetic factors, that are responsible for a critical elevation of the incidence of type 1 diabetes over the recent decades, because such an increase in the proportion of risk genotypes for type 1 diabetes in the population is not likely. In monozygotic twins, a simultaneous occurrence of type 1 diabetes has been highlighted in 23–53% of them.

When compared to other European countries, the Czech Republic has an intermediate but steadily rising incidence of diabetes. Over the recent years, type 1 diabetes mellitus was diagnosed in approximately three hundred Czech children per year (Cinek, 2011).

In Europe, there is a north-south gradient in the disease incidence. The highest incidence is observed in Finland (40.2/100000/year), while the lowest incidence rates are reported by Balkan countries, particularly by Macedonia (3.2/100000/year), with the exception of the island of Sardinia (Karvonen et al., 2000).

In a Swiss clinical trial on experimental gingivitis induced by refraining from oral hygiene for three weeks, there were no differences in the plaque index scores or in the composition of bacterial plaque between the type 1 diabetics and healthy controls, but the diabetics responded to plaque irritation by an earlier developed and more severe gingival inflammation, which corresponded to a significantly higher level of some inflammatory biomarkers in crevicular fluid (Salvi et al., 2010).

Another research performed in a large group of Brazilian child diabetics with a mean age of 13 ± 3.5 years observed gingivitis and periodontitis in 21%, and 6% of the study subjects respectively (Xavier et al., 2009).

Similarly, there are reports of a higher incidence of dental plaque. In children with diabetes, the periodontal breakdown usually manifests in the adolescence (Lalla et al., 2007). Although periodontitis does not belong to clinical manifestations of any type of diabetes mellitus, it is still being labeled as "the sixth chronic complication of diabetes." It has been confirmed that, in individuals with diabetes, there is about a three times higher risk of periodontitis. Thus, diabetes is considered to be a predisposing factor for periodontitis. On the other hand, it was confirmed that there is a negative effect of periodontitis on blood glucose levels. This is due to an increased insulin resistance of tissues in reaction to systemic inflammatory mediators. According to the recent clinical trials, a successful treatment of periodontitis decreased the HbA1c levels (reflecting a long-term diabetes control) of 0.4% (Preshaw et al., 2012).

The effect of proper metabolic control and occurrence of chronic complications of diabetes on the development of periodontal diseases has been confirmed by a number of studies. Recently, a presumption that treatment of periodontitis results in an improved metabolic control of diabetes has been confirmed, although some earlier studies did not support this hypothesis (Salvi et al., 2010). It was thus confirmed that there is an association between poorly controlled diabetes (higher HbA1c levels) and development of periodontitis, even in children with type 1 diabetes. Some studies show relationship between the duration of diabetes and severity of periodontitis (Carollo-Bittel & Lang, 2011).

Hyperglycemia associated during diabetes mellitus can alter immune system in many ways. An increased availability of glucose in the environment of oral cavity increases proliferation of periodontopathic and cariogenic bacteria and increases oral inflammation. Presence of elevated levels of proinflammatory mediators in the gingival crevicular fluid of periodontal pockets in poorly-controlled diabetics, compared to nondiabetics or well-controlled diabetics, resulting in significant periodontal destruction with an equivalent bacterial challenge, has been demonstrated. This exacerbation of the proinflammatory response in diabetics can lead to impaired wound healing, and can amplify connective tissues damage as well (Ryan et al., 2003).

Hyperglycemia also increases the formation of advanced glycation end-products (AGE). The overexposure of proteins (such as collagen) or lipids to aldose sugars induces nonenzymatic glycation and oxidation. These glycosylated products can create complex molecules, reducing collagen solubility and increasing levels of proinflammatory mediators responsible for the degradation of connective tissues. Changes to collagen metabolism result in accelerated degradation of both nonmineralized connective tissue and mineralized bone (Grover et al., 2013).

Prevention and control of oral inflammatory diseases are essential for appropriate prevention and optimal management of diabetic complications (Matthews, 2002).

The prevalence of type 1 diabetes is increasing noticeably among children in many arabic countries. It has been shown that patients with type 1 diabetes mount an exaggerated

gingival inflammatory response to a bacterial challenge compared to that found in nondiabetics. In addition, diabetic patients may have more Gram-negative bacteria than controls. T1D subjects with longer diabetes duration (>2 years) have significantly higher mean clinical attachment loss and more bleeding on probing compared to diabetic patients with shorter diabetes duration. Moreover, T1D with better glycemic control (HbA1c ≤ 9%), have a significantly lower mean gingival index and plaque index and less clinical attachment loss compared to diabetic patients with poor glycemic control (HbA1c > 9%), diabetes duration appearing to play a significant role in the development of periodontitis (Al-Khabbaz et al., 2013).

Several studies have demonstrated that the prevalence, severity and progression of periodontal disease are significantly increased in patients with diabetes (Taylor et al., 2008). Diabetes may influence the periodontium in several ways, including alteration of connective tissue metabolism, modulation of the host immune response and function of immune cells and the upregulation of monocytes, which might lead to uncontrolled release of inflammatory mediators (Mealey & Oates, 2006).

Children with diabetes endure many problems, including oral health complications. Generally, the results confirmed that diabetes is a risk factor for periodontal inflammation in children (Lalla et al., 2007).

A few reports on the relationship between diabetes and periodontal disease have included children and adolescents. In comparison with studies in diabetic children from Europe, diabetic children in some Arabian countries had higher values for the plaque index and gingival index. Interestingly, some studies showed that only 24% of the diabetic children had their first dental visit before the age of 4 years, and a large number of them (44%) had never visited the dentist before (Aren et al., 2003).

Oral health complications might be a great challenge for young subjects affected by diabetes; although the mean clinical attachment loss was not found significantly different, diabetic children had significantly more plaque accumulation and gingival inflammation than nondiabetic children (Lalla et al., 2006).

It has been shown that patients with type 1 diabetes mount an exaggerated gingival inflammatory response to a bacterial challenge compared to that found in nondiabetics (Salvi et al., 2005). Some investigators found that individuals with diabetes and poor metabolic control are at higher risk for more severe periodontitis. Therefore, it might be more useful to use the HbA1c measurements over the previous 12– 24 months and correlate the average with periodontal attachment loss (Lalla et al., 2007).

Current evidence regarding the biological link between diabetes and periodontal disease supports persisting hyperglycemia leading to an exaggerated immunoinflammatory response to the periodontal pathogenic bacterial challenge. Therefore, gingival bleeding at a young age may have some prognostic value for future risk of periodontal disease over a period of time in diabetic children (Nishimura et al., 2007)

Since 2008, the World Health Organization (WHO) presented numerous reports concerning the continuously increasing incidence of insulin dependent diabetes mellitus in the juvenile population, we focused much of our attention on the binomial relationship between insulin dependent diabetes mellitus (IDDM) and periodontal disease within this

age group of individuals, considering both the potential of investigation and prevention of this malady and its complications within the young subjects. The main preoccupations during my research targeted the following aspects:

a. study of the periodontal pathology in child and adolescent, through determination of the role and diagnostic value of certain cytokines measurements, within the complex program of identification, evaluation and treatment of the patients with periodontal disease and unaffected general state (control group) and systemically affected individuals;
b. analysis of impact on periodontal breakdown pathogenesis of the interleukins IL-1β, IL-2, IL-10 and interferon gamma (IFN-γ), and their expression as potential indicators or predictors of diagnostic and evolution of periodontal disease in systemic context.

My contributions to this research direction can be found in the following articles:

1. Foia L**, Toma Vasilica,** Surlin P. Diabetes mellitus impact on periodontal status in children and adolescents. **Chp. 8:** p. 179-197 in Editor J. Manakil; *Periodontal disease – a clinician's guide.* InTech Ed. 2012, ISBN 978-953-307-818-2, DOI: 10.5772/IntechOpen.72268; Link: https://www.intechopen.com/books/periodontal-diseases-a-clinician-s-guide/diabetes-mellitus-impact-on-periodontal-status-in-children-and-adolescents
2. Foia L, **Toma Vasilica:** *Patologia parodontala in diabetul juvenil*. Ed "Gr. T. Popa, Iaşi, 2008.
3. Cioloca DP, Foia L, Holban C, Trandafirescu M, Poroch V, Maxim D, Jipu R, Costuleanu M, **Toma Vasilica**. Systemic diabetic context-induced biochemical periodontal alterations in children. *Rev Chim*. 2016; 67(12): 2409-2412.
4. Foia L, Ungureanu D, **Toma Vasilica**, Zlei M, Indrei A, Haba D, Branisteanu D. Analysis of oral expression of the diabetes-periodontal disease binomial relationship in a juvenile population. *Romanian Review of Laboratory Medicine*, 2008; 13(4): 39-48.
5. **Toma Vasilica**, Surlin P, Cioloca D, Trandafir L, Cozma S, Bogdan M, Botnariu EG, Balan A. Assessment of clinical periodontal modifications in juvenile diabetes. *Romanian Journal of Oral Rehabilitation*, 2015; 7(2): 97-101.
6. Vlad CE, Foia L, Agache CA, Strungaru SA, **Toma Vasilica**, Surdu A, Goriuc A, Florea L. Decompensated diabetes mellitus binomial – emphysematous pyelonephritis and periodontal disease. *Romanian Journal of Oral Rehabilitation*. 2017; 9(4): 97-104.
7. Cioloca DP, Ursarescu I, Martu A, **Toma Vasilica**, Surdu A, Botnariu G, Bogdan M, Caruntu ID. Systemic and periodontal inflammatory burden in children and teenagers with diabetes and clinical correlations. *The Medical-Surgical Journal*, 2015; 119(3): 896-902.
8. Foia L, **Toma Vasilica**, Ungureanu D, Aanei C, Costuleanu, M. Binomial relationship periodontal disease-diabetes mellitus: ethiologic and risk factors. *The Medical-Surgical Journal*, 2007; 111(3):748-753.

II.1.1.2. Materials and Methods

- ***Selection of subjects by group of study and criteria for biological samples collection***

Our study has been carried on a numer of healthy subjects and those affected by diabetes, with periodontal involvment. The research has been carried out on 84 subjects, age 6 – 18 years, divided into two groups, both with several degrees of periodontal alteration: the control group which consisted in 42 non-diabetic subjects who did not suffer from any systemic disease and 42 IDDM subjects.

The age of the subjects was considered criteria for inclusion in sub-groups.

According to the age, prepuberal (6-10 years old), pubertal (11-14 years) or juvenile age (15-18 years old), and metabolic control of the disease, the subjects were evaluated and divided into subgroups. The diabetic group enrolled in this study comprised 21 well-controlled diabetes cases (glycosylated hemoglobin levels ≤7%) and 21 patients with poorly controlled diabetes (glycosylated hemoglobin levels >7%).

All subjects were submitted blood collection, GCF sampling and clinical periodontal index evaluation. Data on blood glucose, lipid profile and glycosylated hemoglobin (HbA1c) were collected from the medical records.

Considering the bivalent nature of the relationship between DM and PD, the evaluation of the gingival fluid comprised records of several immune-chemical inflammatory mediators: interleukin 1β – Il-1β, IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ, in parallel with serum mediator determinations. Total amounts and concentrations of serum and gingival crevicular cytokines were analyzed by enzyme-linked immunosorbent assay and flow cytometry.

Diabetic patients were recruited from the Metabolic and nutrition diseases department of the University Children Hospital “Sf. Maria” Iasi, and selected based on the following criteria: aged between 6 and 18 years old, diagnosed with type 1 DM. Patients were excluded if they had non-type 1 diabetes, any inflammatory diseases, liver or renal impairment (depending of the blood creatinine levels), a periodontal treatment in the last 6 months prior to the assessment, any severe pathology of the teeth or were receiving medication that could influence the studied parameters (corticosteroids, antibiotics). The age matched control group was selected among the non-diabetic individuals that followed regular treatment in the dental unit of the Pediatric Dental Clinic. Informed consent was obtained in all cases, the local ethics committee approving the protocol deemed to conform to the guidelines issued in the Helsinki Declaration.

The study included only the children and adolescents having the parents and grandparents from european (north caucasian) race, to reduce the genetic heterogeneity. Meanwhile, the study excluded from the beginning the children and adolescents with a recent history of hepatitis or antibiotics.

From a clinical point of view, the periodontal status was assessed by clinical evaluations of:

- plaque index (PI),
- papillary bleeding index (PBI)

- clinical attachment loss (CAL)

All the data have been collected in and correlation with the degree of metabolic control (levels of glycemia and glycosylated hemoglobin).

The mentioned periodontal parameters were evaluated in a randomized half mouth examination on six sites of each tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual) by a calibrated examiner. The level of oral hygiene was estimated with a plaque index – Quigley Heine index (based on the score from 0 to 5) (Silness and Löe, 1964). The scores of the plaque index were calculated according to the formula: per person = sum of individual scores/number of teeth present for each person, subsequently, the group scores being subtracted. Other clinical records consisted of papillary bleeding index evaluation, based on gentle probing and clinical attachment loss determinations of the total teeth in the mouth by periodontal probe exploration. PBI score (Saxter and Muhleman) was recorded based on four different grades of bleeding intensities subsequent to careful probing.

- **Gingival crevicular fluid and serum sampling**

Collection and analysis of GCF represent noninvasive methods for the evaluation of host response in periodontal disease. Gingival crevicular fluid samples were obtained from the mesiobuccal site of every tooth (excluding third molars) from two randomly selected contralateral quadrants. Consecutive plaque evaluation and following isolation of the site with cotton rolls, supragingival plaque was removed, and the tooth air dried. GCF sample was collected on periopaper strips (Periopaper®, Amityville, NY) gently inserted 1–2 mm subgingivally, into the periodontal pocket. Gingival fluid volume was assessed using an electronic device, Periotron 8000® [Oraflow Inc., Plainview, NY].

Collected samples were immediately placed into sterilized plastic tubes on ice, shipped to the laboratory and stored at −80°C till the day of determination. GCF samples were always collected prior to clinical measurements and samples contaminated with blood were discarded. Using the venipuncture technique, approximately 5 ml of venous blood was also drawn from the antecubital vein, using the vacutainer system [Becton Dickinson, NJ, USA], and analyzed for the lipid and carbohydrate metabolic profile. The degree of metabolic control was evaluated considering the glycosylated hemoglobin values (HbA1c), measured by high performance liquid chromatography (HPLC).

Good metabolic control has been considered when HbA1c $\leq$ 7%, while poor control was defined as HbA1c > 7%, (American Diabetes Association), normal values being considered for HbA1c < 6%.

- **Measurement of the gingival crevicular fluid (GCF) volume**

Considering that the two pathologies, diabetes and periodontitis are both relying on inflammatory grounds, we considered that an important parameter in assessing the interrelation between them would be the estimation of the degree of inflammatory process through measurement of the small volumes of the local gingival fluid. Therefore, after isolating the tooth with cotton roll, GCF was collected by placing filter paper strips

(Periopaper, Pro Flow, Amitywille, NY) into the mesial sulcus of the permanent incisor and premier molars until mild resistance was cented, and left in place for 30 seconds. The volume of the sample on the filter paper strips was measured using a calibration unit (Periotron 8000, ProFlow).

- **Measurement and quantification of cytokines using multiplex cytometric bead array**

Serum and local gingival fluid cytokine levels were determined using the high sensitivity human CBA cytokine multiplex [Cytometric Bead Array®, BD Pharmingen, San Diego] for flow cytometry. Prior to assay, GCF samples were eluted into 50 µl of the assay buffer by vortexing for 30 minutes and further 10 minutes-centrifugation at 8,000 rpm. Flow cytometry is an investigation method that allows various cells sorting according to size, granularity, and specific markers expression.

The method used for determining the amounts of cytokines both in plasma and gingival crevicular fluid was flow cytometry. The levels of interleukins IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were measured by means of a kit multiplex: CBA (Cytometric Bead Array/Human Th1/Th2 cytokine array) for flow cytometry.
Meanwhile, plasma was separated from the same patients to determine the concentrations of pro- and anti-inflammatory cytokines, synthetized and released by leukocytes during the host inflammatory response.

Cytometric investigation of cytokines has substantial advantages compared to ELISA immunoassay method, allowing simultaneous detection of multiple cytokines, fast and with very small sample volumes (50µl). CBA kit contains microspheres coated on the outside with anti-cytokine monoclonal antibodies. Each type of microsphere has a characteristic fluorescence level detectable on third channel (FL 3) of the cytometer.

Detection of cytokine amount is performed through the second category of anti-analyte antibody, marked with a fluorescent protein, phycoerythrin, whose fluorescence is detectable on channel FL 2.

The FACS Caliber (BD Biosciences, San Jose, CA, USA) monitors the spectral properties of the beads to distinguish the different antigens, simultaneously measuring the amount of fluorescence associated with phycoerythrin and reported as median fluorescence intensity. The concentrations of the assessed samples analytes were estimated using a standard curve obtained following the manufacturer's instructions, by testing standard samples included in the kit and expression as pg/ml.

Periodontal parameters of subjects according to the age group were described by means of standard deviation and analyzed by analysis of variance (ANOVA).

- **Clinical examination protocol**

Clinical data were collected from 6 sites per tooth for visible plaque, papillary bleeding index, and CAL. The levels of each inflammatory mediator were measured for up to 14 GCF samples per subject and expressed as pg/ml. Interactions between variables were studied using Pearson's correlation. The Mann–Whitney *U* test was used to compare

values between groups. Paired non-parametric (Wilcoxon) *t* tests established significance for cytokine level within gingival fluid and serum from the same individual, while $p<0.05$ established significance.

Dental examination regarded the overall health of fully erupted permanent teeth (third molars were excluded) and the surrounding tissues. The level of oral hygiene and periodontal tissues inflammation and disorder were estimated by the following:

- Plaque index (PI); according to (Silness and Loe, 1964) each site was given a score from 0 (absence of plaque) to 3 (abundant soft matter within gingival pocket, margin and adjacent surfaces). Score 1 defines the existence of a thin film adherent to the free gingival margin and adjacent tooth area; score 2 - moderate accumulation of soft deposits within gingival pocket and gingival margin and/or tooth surface.
- Papillary bleeding index (PBI); separates four different degrees of bleeding, subsequent to careful probing into papillary region: 0 - no bleeding; 1 – one single bleeding point, 2 – a fine line of blood or several bleeding points become visible at the gingival margin, 3 – the interdental triangle is filled with blood, 4 – profuse bleeding after probing. The bleeding value was given by the sum of the recorded scores and PBI by dividing the bleeding value to the total number of examined papilla.
- Attachment level (CAL), the distance from the cementoenamel junction to the base of the periodontal pocket, a measure of the amount of alveolar bone lost due to periodontal disease, was measured to the nearest millimeter using a North Carolina periodontal probe. Measurements of 1–2 mm were considered to be slight, 3–4 mm moderate, and ≥5 mm severe (Costa et al., 2007).

All clinical indicators were evaluated on Ramfjörd teeth level, at mesio-vestibular sites.

- **Evaluation of immune-inflammatory response, through local and systemic enzyme and cytokine estimation**

Among the biological fluids, we selected the study of GCF because of its numerous advantages: unlike the blood and saliva, convenient samples from specific sites, which contain components, derived both from host and bacterial plaque can be used. Given that the collection method affects the amount of obtained gingival fluid, we used the less aggressive method, with the introduction in gingival sulci of strips with standardized sizes, after the rigorous control of bacterial plaque, isolation from saliva with cotton rolls and dry of the gingival sulci.

According to Brill & Krasse (1958), the strips were inserted subgingivally, from vestibular to oral, at mesial facet of the Ramfjord teeth (16, 21, 24, 36, 41, 44 or its neighbors) and left in place for 30 seconds. Temporary teeth were not taken into consideration unless their successors were erupted. The strips were placed in cold phosphate buffer pH = 7.4 (at 4ºC), stirred for 5 minutes using a vortex and then the content was divided into 2 tubes, for IL-1β and aspartate transaminase - AST determinations. The samples were immediately prepared or stored at -70º C in plastic containers resistant to that

temperature.

The biological parameters also analyzed within the fluid that bathes the virtual space between gingiva and teeth, during evaluation of the binomial relationship IDDM – periodontal breakdown, were the intracellular transaminase AST and interleukin IL-1β.

For gingival fluid AST activity determination, we used spectrophotometric method, on a Hewlett-Packard spectrophotometer and the INIFINITY® AST test (Sigma), using the manufacturer's protocol. The obtained data were directly expressed by the soft in U/l AST**.** Gingival fluid IL-1β investigation was instrumented by enzyme-linked immunosorbent assay (ELISA) using Human Interleukin-1β - hIL-1β, in accordance with the described protocol.

- **Statistical evaluation**

The GCF levels of AST and IL-1β were expressed as average and compared with the control group. The statistical differences between the interested values corresponding to our studied groups were tested using the t-Stu-dent test, One-Way ANOVA completed by Kruskal-Wallis test, for GCF IL-1b, AST, and clinical indicators (PI, BPI and CAL) investigation. The differences were considered statistically significant for level of significance (p) lower than 0.05, corresponding to a level of confidence of 95%. Moreover, clinical indicators and AST were separately calculated related to age (prepuberal: 6-10 years, puberal: 11-14 years, juvenile: 15-18 years), on incisive, pre-molar and molar level respectively.

II.1.1.3. Results

The main target was evaluation of the changes in the clinical parameters of periodontal damage in the context of systemic insulin dependent diabetes in children and adolescents, correlated to IDDM metabolic control and also inflammatory process.

- ***The impact of diabetes mellitus on the periodontal status in children and adolescents***
  - **Oral hygiene levels**

We used the Quigley Heine index to asses the level of oral hygiene for all the patients based on the score from 0 to 5.

Our results showed a high incidence of values in the 2-3 range for the non-diabetes group (ND) compared with the distribution of values in other groups. In the analysis presented in Table II.1.1.1, statistical indicators display a high average of plaque index in poorly controlled diabetic patients (3.293 ± 1.06) compared to mean values calculated for the non-diabetes group (2.995 ± 0.58) and individuals with well-controlled diabetes (2.881 ± 0.857), respectively. Standard deviation registered the minimum value for the nondiabetics (SD = 0.58) while for the group with poorly-controlled diabetes, standard deviation reached a maximum value (SD=1.06).

Table II.1.1.1. *Statistical indicators of Quigley Heine Index for studied groups, according to age - (ND), good control - and poor control in DM groups.*

QH Index / Group	Mean	Std. Dev	Min	Max	Q25	Q50	Q75
Pre puberal age: 6-10 years							
ND	3.176	0.500	2.500	4.133	2.830	3.058	3.50
Good control DM	3.132	0.736	2.000	4.000	2.660	3.500	3.50
Poor control DM	2.830	0.626	2.000	3.660	2.330	2.830	3.33
Pubertal age: 11-14 years							
ND	2.734	0.445	2.330	3.660	2.330	2.660	3.00
Good control DM	3.125	0.888	1.833	4.133	2.660	3.000	4.00
Poor control DM	3.076	0.884	1.833	4.133	2.330	3.080	4.00
Juvenile age: 15-18 years							
Martor	3.036	0.668	2.166	4.000	2.500	3.000	3.66
Good control DM	2.356	0.679	1.500	3.500	1.833	2.000	3.00
Poor control DM	3.925	1.207	1.833	5.000	3.660	4.133	5.00

.

ANOVA test used to compare by analysis of variance the mean plaque index values corresponding to studied groups, highlighted the significant difference between mean values corresponding to the groups and subgroups ($p = 0.013$, $p<0.05$). Critical difference existed also between the values corresponding to the ND and poorly controlled DM individuals, the significance level (p) corresponding to 95% confidence interval being 0.0451. Statistically significant difference was record between the plaque index levels within the two diabetes subgroups: good control versus poor control DM, as well - $p=0.003$ ($p<<0.05$, CI=95%).

Quigley Heine Index can be properly evaluated in the studied groups taking into account the patient's age. The maximum standard deviation was found in the group of patients aged 15-18 years (juvenile period), significant differences being registered in this group between average values of nondiabetics and diabetics (Table II.1.1.1). Maximum values (QHI= 5) were recorded for the juvenile group (15-18 years old), in patients with poorly controlled IDDM. Considering the age intervals, comparative statistic studies of the oral hygiene parameter highlight the most significant differences among the juvenile age individuals from all studied groups ($p<<0.05$).

- *Papillary Bleeding Index (PBI)*

Bleeding index diplayed different values in the two populations. Hence, the non-diabetes group stands 0.5 minimum and 2.66 as maximum values, lower than those for patients with poorly-controlled diabetes (PBI min = 1, max = 4.66). Large variations recorded among the bleeding index values in the group with poor metabolic control are also highlighted by the large standard deviation (SD = 0.97).

As displayed in Table II.1.1.2, the average PBI in poorly controlled diabetes is 2,964, almost two times higher than in the non-diabetic group (PBI = 1.56) and 1.75 times higher than in the well balanced diabetic disease group (PBI = 1.69), $p<<0.05$. Statistic analysis revealed no significant differences between PBI values of the systemically unaffected population and diabetic subgroup with good metabolic control ($p = 0.58$), while significant differences were registered between the two subgroups of diabetics ($p = 0.000018$, $p<<0.05$).

Statistic analyses on PBI correlated to age stages highlighted minimum values in the ND group within juvenile age (PBImin=0.50) and maximum values (PBImax=4.66) recorded in the prepubertal age group of subjects affected by poorly-controlled diabetes (Table II.1.1.2).

Important and critical differences were recorded for the mean PBI values for all groups of patients divided per age groups ($p<0.5$). For prepubertal stage (6-10 years) mean PBI did not registered significant differences between ND and good metabolic control patients ($p>0.5$), while for pubertal stage significant differences were recorded across all studied groups (ND, good-control and poor-control DM).

Considering the juvenile period (15-18 years), average PBI was higher in poor controlled diabetes compared to mean values of well metabolically balanced diabetics and of ND, the difference being statistically significant ($p<0.5$).

Table II.1.1.2. *Statistics on papillary bleeding index (PBI) for the studied groups, according to age, in the non-diabetes group, poorly-controlled and good metabolic controlled diabetic children and adolescents.*

PBI / Group	Mean	Std. Dev	Min	Max	Q25	Q50	Q75
Pre puberal age: 6-10 years							
ND	1.914	0.472	1.330	2.660	1.580	1.830	2.250
Good control DM	2.130	0.899	1.330	3.660	1.660	2.000	2.600
Poor control DM	3.039	1.187	2.000	4.660	2.165	2.748	3.913
Pubertal age: 11-14 years							
ND	1.235	0.593	0.660	2.330	0.660	1.330	1.500
Good control DM	2.650	0.850	1.330	3.660	2.600	2.660	3.000
Poor control DM	2.775	0.777	2.000	3.660	2.000	2.665	3.660
Juvenile age: 15-18 years							
ND	1.499	0.819	0.500	2.660	0.833	1.500	2.330
Good control DM	2.747	0.457	2.000	3.300	2.330	3.000	3.000
Poor control DM	3.132	1.260	1.000	4.000	3.000	3.660	4.000

- *Clinical attachment loss (CAL)*

The highest incidence of increased clinical attachment loss along with the most elevated mean value were recorded in poorly-controlled diabetics (CAL = 1.053 mm, Table II.1.1.3).

Significant differences were recorded between the two subgroups of DM children and adolescents and between the groups of non-diabetes and good metabolic control DM, respectively (p=0.002).

For a description of the groups included in the study based on loss of attachment, Table 3 presents statistical indicators that define the characteristics of the groups in terms of this clinical indicator. For pre-puberal stage no real attachment loss was registered in all groups of subjects enrolled in the study. The puberal phase recorded a slight increase in the CAL levels, with maximum values up to 2mm, and 0.5mm as average.

Quartile analysis (Q75) indicated that 75% of the children belonging to this age group presented mean CAL levels below 1 mm. Individuals aging between 15-18 years old recorded different values, with minimum CAL=0mm and peak CAL=4 mm, statistic analysis highlighting mean values below 2.3 mm for 75% of non-diabetics, while 75% of poor controlled SM subjects of this age group presented CAL up to 3.5 mm (Table II.1.1.3). Moreover, standard deviation was also higher for this age population compared to the others.

Table II.1.1.3. *Statistic indicators of clinical attachment loss (CAL-mm) for studied groups, according to age.*

CAL(mm) **Group**	**Mean**	**Std. Dev.**	**Min**	**Max**	**Q25**	**Q50**	**Q75**
Pre puberal age: 6-10 years							
ND	0.000	0.000	0.000	0.000	0.000	0.000	0.0
Good control DM	0.000	0.000	0.000	0.000	0.000	0.000	0.0
Poor control DM	0.000	0.000	0.000	0.000	0.000	0.000	0.0
Pubertal age: 11-14 years							
ND	0.000	0.000	0.000	0.000	0.000	0.000	0.0
Good control DM	0.000	0.000	0.000	0.000	0.000	0.000	0.0
Poor control DM	0.500	0.786	0.000	2.000	0.000	0.000	1.0
Juvenile age: 15-18 years							
ND	1.033	1.545	0.000	4.000	0.000	0.000	2.3
Good control DM	0.786	1.280	0.000	3.000	0.000	0.000	2.5
Poor control DM	2.560	1.447	0.000	4.000	2.300	3.000	3.5

- **Periodontal biochemical alterations induced by diabetes in young subjects**

The inflammatory mediator profile in human whole blood and gingival fluid was characterized in more detail. Whole blood and crevicular fluid were collected from all subjects enrolled in the study, according to the previous mentioned protocol, and analyzed for IL-1β, IL-2, IL-4, IL-10, TNF-α and IFN-γ production. The degree of local and systemic inflammatory response was assessed by multiplex flow cytometry blood and

gingival fluid cytokines level determinations. A significant interindividual variability in the amounts of inflammatory mediators secreted during the association of the periodontal breakdown with systemic alteration was observed for all the mediators tested.

As shown in figure II.1.1.1, diabetes mellitus elicited a significant increase ($p<0.05$) in local secretion of IL-1β.

The lowest mean local interleukin 1β value was recorded in the systemically healthy population, the diabetic status associating a considerable increase in gingival fluid interleukin levels.

In addition, systemically healthy patients with gingivitis recorded the lowest gingival fluid IL-1β level, a significant elevation of this mediator being associated to IDDM children and teenagers. Moreover, IL-1β, IL-2 and IFN-γ analysis according to the values of HbA_{1C}, revealed the existence of a statistic significant positive correlation betwen the measured parameters (Pearson test, r=0.73; 0.65 and 0.71 respectively), thus reflecting important elevations of cytokine levels induced by impaired metabolic balance of the diabetic young population.

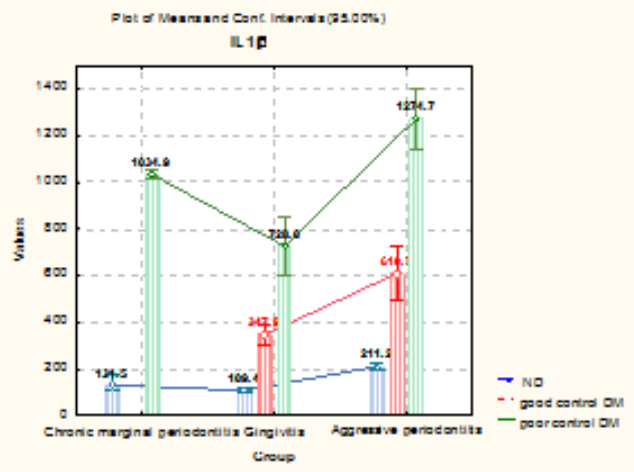

Figure II.1.1.1. *Levels of gingival fluid IL-1β in the studied groups*

Interferon gamma (IFN-γ) is an immunoregulatory cytokine which by activated through its receptor can trigger the activation of inflammatory events, which are the basis of periodontal dimpairment. Like stem cells from other tissues, the stem cells from the periodontal ligament possess immunomodulatory capacity and are regulated by some cytokines such as interferon-gamma, that is why we choosed to measure the level of this mediator in the gingival fluid of children affected by the diabetic disorder, as well.

Serum IFN-γ in diabetic children recorded moderate values compared to that of ND, and significantly higher levels in gingival fluid (Figure II.1.1.2). While IL-10 gingival fluid secretion was enhanced in some diabetic subjects with good control of the metabolic disorder, the most common elevated levels were present in the serum of systemically unaffected group (Figure II.1.1.2 and Figure II.1.1.3).

Interleukin-10 inhibits cytokine production by activation of T- helper (Th1) clones, due to inhibitory effect on monocyte and macrophages (Bastos et al., 2011). This

antiinflamattory cytokine specifically modulates the expression of cytokine of myeloid origin, impairinh thus activation and maintenance of immune response in periodontitis.

In our study, the anti-inflammatory IL-10 registered a decreased average level of blood and GCF secretion in the diabetic population, with significantly differences between the two systemically affected subgroups. Moreover, considering IL-2 level, there was a very low secretion registered for diabetic subjects with periodontal impairment, both in the blood and in the locally secreted fluid, probably determined by a local production of a blocking factor that induces this specific profile.

Cytokine concentrations from all subjects were compared between the control and diabetic groups (considering their metabolic status of basic disease), and correlated with the degree of periodontal alteration (gingivitis or periodontitis). Gingivitis (inflammatory process limited to the mucosal epithelial tissue surrounding the cervical portion of the teeth) was assessed in patients displaying signs of inflammation (rubor, dolor, calor, tumor) strictly localized at mentioned area and bleeding tendency.

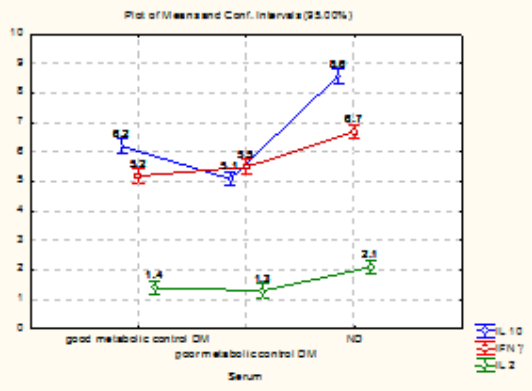

Figure II.1.1.2. *Serum levels of IL-2, IL-10 and IFN-γ in the studied groups.*

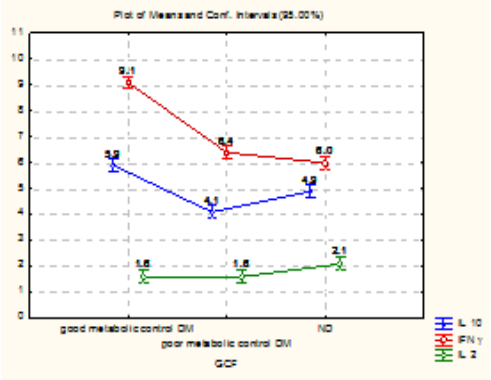

Figure II.1.1.3. *GCF levels of IL-2, IL-10 & IFN-γ in the studied groups.*

A higher prevalence of periodontitis was correlated with the degree of glucose metabolic imbalance. Following clinical evaluations and dental indicators, criteria for statement of periodontitis were chosen in agreement with the European Workshop in Periodontology (Tonetti & Claffey, 2005): at least two teeth with at least one site with attachment loss >2 mm.

The above data analysis and reflected in Table II.1.1.4 emphasizes that systemically healthy patients with mildest form of periodontal alteration (gingivitis) recorded the lowest gingival fluid IL-1β concentrations (109.36 ng/ml). Furthermore, the values of the gingival cytokine in the aforementioned group increased significantly concomitantly with the severity of the disease, an approximately 2-fold elevation within gingival fluid of periodontitis subjects being recorded (211.16 ng/ml).

Table II.1.1.4. *IL-1β concentrations in gingival fluid depending on systemic status and periodontal degree alteration*

GROUP IL-1β		Diagnosis	Nr. cases	Mean [ng/ml] IL-1β	Std. Dev	Min	Max
CONTROL		Gingivitis	22	109.36	41.88	0.00	140.00
		Periodontitis	2	211.17	12.02	199.00	230.00
Total			24	117.85	49.18	0.00	230.00
IDDM	GOOD METABOLIC CONTROL	Gingivitis	15	347.89	133.59	0.00	510.00
		Periodontitis	2	610.67	106.57	515.00	785.00
	Total		17	378.80	155.41	0.00	785.00
	POOR METABOLIC CONTROL	Gingivitis	9	727.96	311.13	0.00	905.00
		Periodontitis	6	1274.67	52.54	1215.00	1314.00
	Total		15	1001.31	300.69	0.00	1314.00

A significant augmentation of the GCF IL-1β is associated to IDDM. The concentrations of this chemical mediator were situated on a foreshore from 347.89 ng/ml in well metabolically controlled IDDM subjects displaying gingivitis, to 1274.67 ng/ml in poorly controlled diabetics with periodontitis (Figure II.1.1.4).

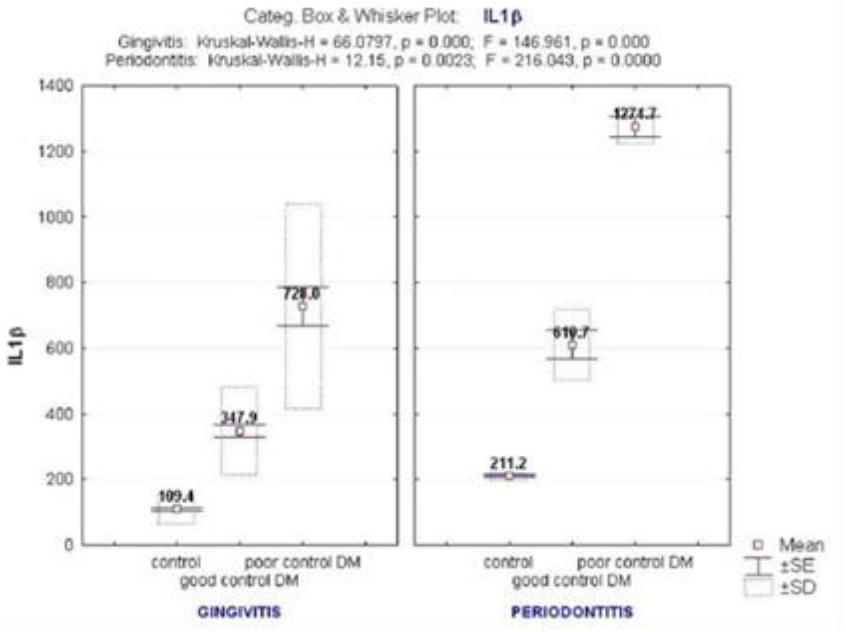

Figure II.1.1.4. *Comparative analysis of gingival fluid IL-1ß concentrations (ng/ml) in normal and diabetes mellitus subjects displaying gingivitis and periodontitis.*

The comparative analysis of the mean IL-1β levels in GCF was referred for both, diabetic and non-diabetic groups, on the clinical importance of the periodontal breakdown. Thus, most of the individuals displayed a mild form of periodontal alteration (gingivitis), while severe periodontal injury (periodontitis) was diagnosed mainly in the diabetic children with poor metabolic control, and less in nondiabetics and well metabolically controlled diabetics (Table II.1.1.4).

The study results recorded correlations of periodontal clinical status with diabetes mellitus biological parameters as well as with host inflammatory response. Therefore, considering the interferon IFN-γ in the plasma of patients, it recorded moderately or significantly increased values as compared to that of the control.

It is also important to observe that the levels of IFN-γ recorded in gingival crevicular fluid are significantly lower in patients with insulin-dependent diabetes mellitus (Figure II.1.1.2, II.1.1.3 and Figure II.1.1.5). Such a behavior could be explained by alterations in oral microenvironment induced by the significantly higher values of gingival crevicular fluid glucose and urea in patients with diabetes.

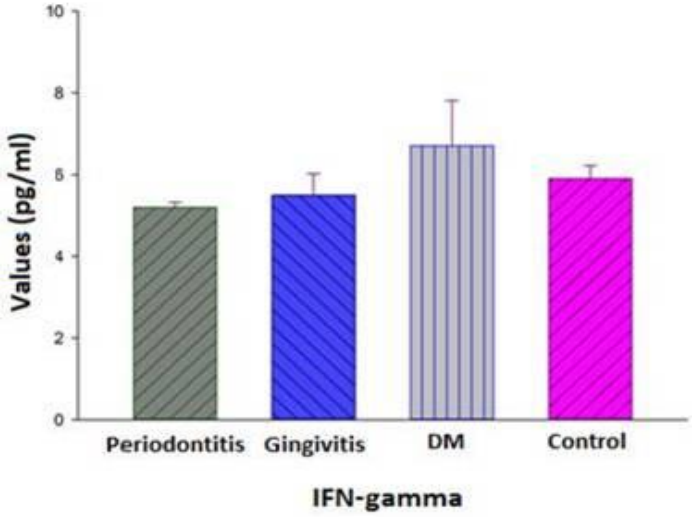

Figure II.1.1.5. *IFN-γ in the plasma of patients with insulin-dependent diabetes mellitus (IDDM) is found at a level that is moderately or significantly increased as compared to that of control.*

The changes in oral micro-environment create a favorable bacterial aggression with altered host immune response to periodontal pathogens. IL-10 associates an average level of secretion in blood (Figure II.1.1.2 and Figure II.1.1.6), being decreased in the group with severe periodontal disease (the values in gingival crevicular fluid are inconsistent throughout the studied groups).

IL-10 suppresses the production of metalloproteinases, while increasing the synthesis of tissue inhibitors of metalloproteinases in macrophages; Moreover, it stimulates production of osteoprotegrin, which consequently inhibits bone resorption by preventing receptor activator of nuclear factor kappa-B ligand RANKL engagement. IL-10 can be a protective cytokine in periodontal disease and regulates pro- inflammatory cytokines, including those implicated in alveolar bone loss and hence, this reduction in IL-10

secretion could play a role in driving the way for oral tissues toward the degradation in juvenile diabetes population.

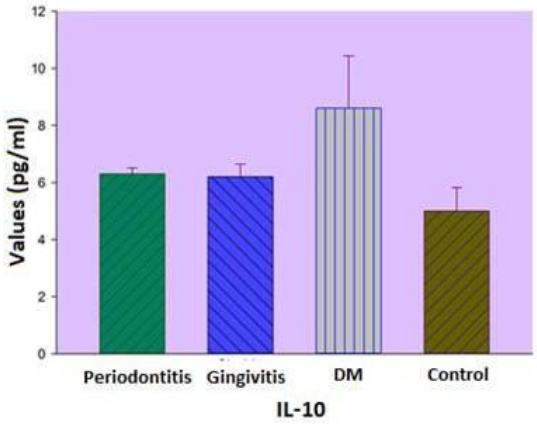

Figure II.1.1.6. *IL-10 associates an average level of secretion in blood, being decreased in the group with severe periodontal disease*

Understanding the role of interleukin IL-2 in the etiology of T1D requires knowledge of the regulation of—as well as the structural and functional consequences of—IL-2 binding to its receptor, the levels of this cytokine being found reduced both in the periphery and in gingival crevicular fluid (Figure II.1.1.3. and Figure II.1.1.7). These data suggest the possible existence of a factor secreted at the local level and able to supress the secretion of this T-cell proliferation factor by lymphocytes and macrophages, especially in diabetic patients with periodontal alteration. Moreover, downstream cellular response to IL-2 depends not only upon surface expression of the receptor but also upon local cytokine concentration, target cell population, and modification of the various response elements in this complex pleiotropic signaling pathway.

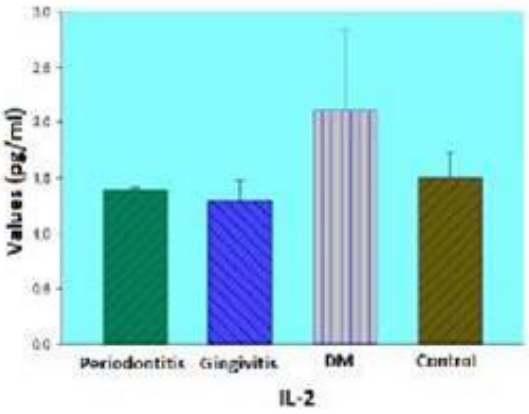

Figure II.1.1.7. *The levels of IL-2 are small in the periphery (plasma)*

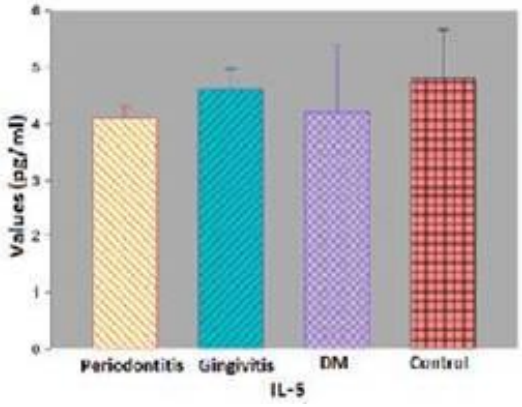

Figure II.1.1.8. *The levels of IL-5 in periphery are reduced, although highest as compared to IL-4 or IL-2*

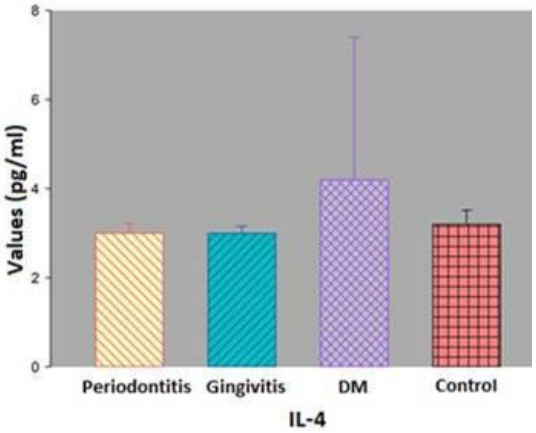

Figure II.1.1.9. *IL-4 levels of secretion in plasma for all groups of patients*

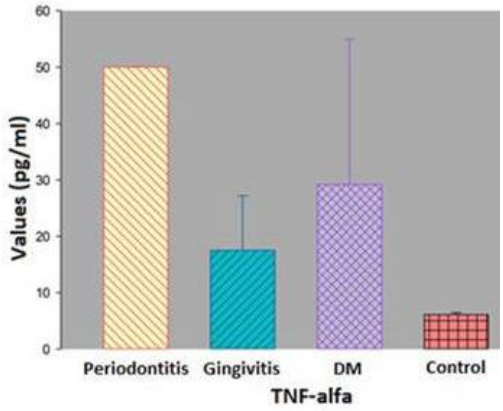

Figure II.1.1.10. *The differences of TNF-α secretions between groups of diabetic patients with or without periodontal alterations in plasma*

We found relatively low IL-4 and IL-5 levels of secretion both in plasma and gingival crevicular fluid for all groups of patients, although a little bit larger in the case of IL-5 (Figure II.1.1.8. as compared to Figure II.1.1.9.).

Some studies have demonstrated the deficiency in interleukin IL-4 production by T-cells mediates immune dysregulation and was proposed to be a causal factor of type 1 diabetes in non-obese but diabetic mice, very probably operating via different mechanisms than IL-10 to restore tolerance during the development of diabetes. The pattern of distribution is quite irregular in all the experimental groups (Mi et al., 2004).

The values of IL-4 in gingival crevicular fluid of our subjects with periodontitis were significantly higher than those of the control. The differences in cytokine secretions between groups of diabetic patients with or without periodontal alterations are more apparent in the plasma, considering TNF (Figure II.1.10), as well as IL-4, IL-5, and IL-2 (the latter one with absolute values of secretion lower than those of IL-4 and IL-5).

The proinflammatory cytokine tumor necrosis factor-alpha is produced by adipocytes, and its blood concentration is elevated in patients with higher weight and declines with weight loss. Studies have demonstrated that TNF- α decreases insulin action via its specific receptor and thus, it exacerbates insulin resistance. Moreover, monocytes/macrophages produce mount TNF-α. Thus, TNF-alpha, produced from monocytes due to inflammatory diseases may have an supplimentary influence on insulin sensitivity to TNF-α.

- **Intracellular enzyme assessment**

The determination of biomarkers in saliva and other oral fluids is becoming an important part of laboratory diagnostics and the prediction of not only periodontal, but also other tissue and organ diseases. Biomarkers in the gingival fluid (e.g., enzymes, protein markers, or oxidative stress markers) can be used for activity determination and for periodontal disease prognosis. GCF contains also many markers which can predict the risk

of certain diseases (e.g., diabetes mellitus, cardiovascular, oncology, endocrinology, and psychiatric diseases). The study of gingival fluid components clearly shows the relationship of periodontal breakdown and diseases of distant systems, organs, or tissues.

The comparative analysis of the mean activities of aspartate amino transferase - AST, based upon dental pattern, among our studied groups, is presented in figure II.1.1.11.

GCF is a very complex system which includes not only both its own components and sulcular fluid components, microorganisms, and products of inflammation ongoing in periodontium, but also metabolites and signal molecules accompanying remote processes. Some components of this local fluid can come from multiple sources, such as proteolytic enzymes. These can come not only from polymorphonuclear leukocytes and periodontal microorganisms, but also from the bloodstream. It is similar to the products of inflammation which may also have both local and systemic origin.

AST is used as a surrogate marker for many epidemiological studies, and also one of the longest studied markers of inflammation. It belongs among transaminases which have been investigated for many years in clinical biochemistry. The enzyme catalyzes the transamination of glutamic acid to oxaloacetic and aspartic acids. During inflammation, AST tissue level rises; it gets into the blood plasma and also by diffusion through salivary glands into saliva. During periodontal inflammation, it also passes into sulcular fluid and then into saliva. AST levels are significantly and positively correlated with the intensity and extent of periodontal inflammation.

As displayed in figure II.1.1.11, a 3.33-fold increase of the mean incisive AST activity in the poorly controlled IDDM group compared to control group (144.93 vs. 43.5) and of 1.43 compared to good metabolic controlled IDDM subjects has been registered. Elevated liver enzyme activity may also reflect inflammation, which impairs insulin signaling

The liver is an important site for insulin clearance and production of inflammatory cytokines, in our study, at the premolar level, children and teenagers with diabetes (regardless of their metabolic control) recording significantly more periodontal modifications, as mean enzyme activities registered higher records comparative to controls. Therefore, compared to control, significant increase of 2.18-fold of AST activity among poor controlled IDDM (100 ± 26.7 vs. 45.8 ± 19.76) and less of 1.98 times, among good-controlled IDDM (90.94 ± 24.4 *vs.* 45.79 ± 19.76) were recorded (Figure II.1.1.11).

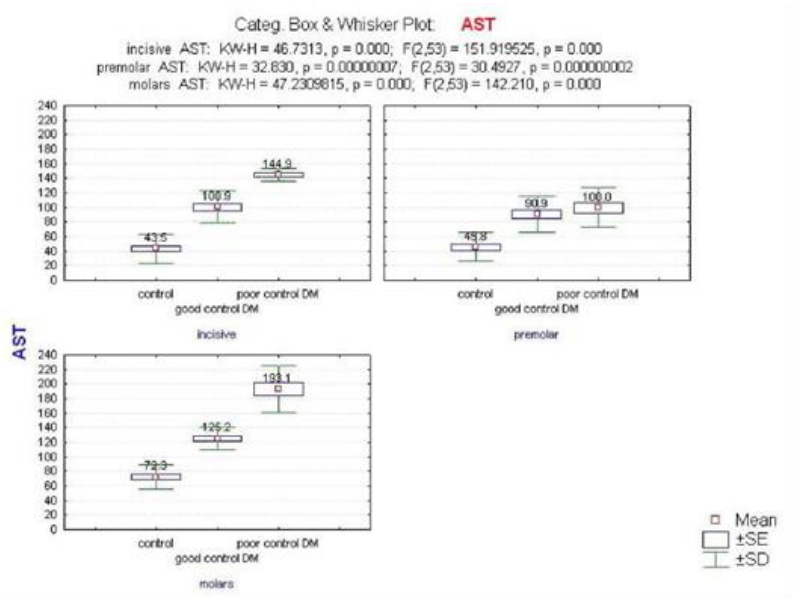

Figure II.1.1.11. Mean AST activities (U/l) based upon dental pattern in diabetes mellitus patients compared with control.

The AST activities around molars recorded higher SD in the group with poorly controlled IDDM compared to the other groups (Figure II.1.1.11), indicating therefore very high variations of the enzyme activities within the mentioned group (min: 143, max: 235). Comparative analysis based on dental site, points out marked AST elevations in the aforementioned subjects around molars.

Among young subjects with poor control of their metabolic disease, the AST activities remained higher for all dental patterns when comparing to well-controlled diabetics and control. Thus, poor controlled IDDM recorded AST activities between 100.0 ± 26.7 and 193.1 ± 31.4, approximately 3-fold magnitude than control group (between 43.50 ± 19.15 and 72.25 ± 16.80) and 1.5 times higher than levels of well controlled IDDM group (ranging between 90.94 ± 24.91 to 125.18 ± 15.57) (Figure II.1.1.11).

Moreover, based upon age, comparative dental pattern analysis of intracellular enzyme marker within GCF revealed significant differences in and between the studied groups (Figure II.1.1.12). There is a general increase of mean AST activity with age, with significant elevation of enzyme activity in puberal and juvenile period.

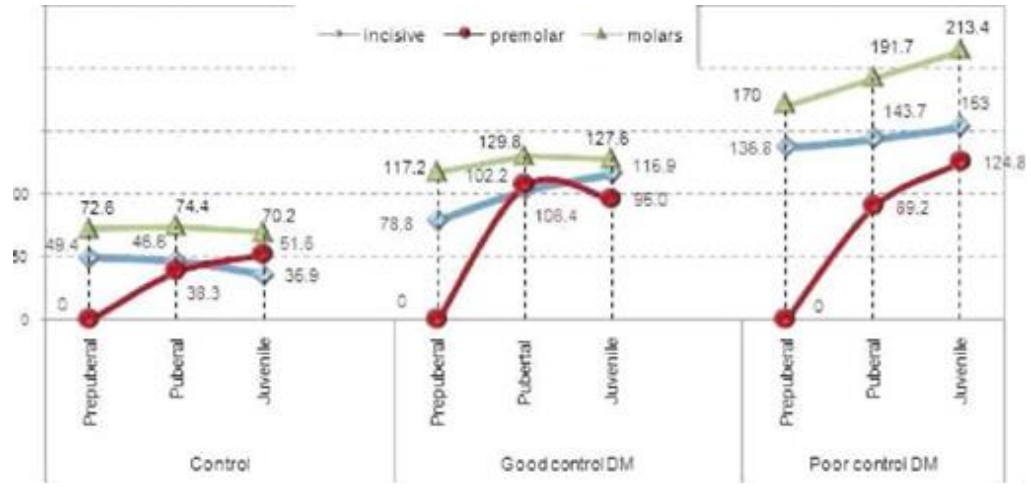

Figure II.1.1.12. Comparative analysis of gingival fluid AST activities (U/l) based upon dental pattern and age, in diabetes mellitus patients compared with control.

Enzymes, specific and nonspecific proteins, antibodies, and other substances are among the potential salivary biomarkers of periodontal and certain distant tissue diseases. Therefore, gingival fluid became the topic of interest among experts in proteomics, research of sequential composition of individual proteins.

- **Periodontal modifications in juvenile diabetes - clinical assessment**

The systemic disorder exerts the effect in a generalized manner and so also affects the occurrence and management of the periodontal conditions. One of such systemic conditions playing an important role in the etiology of periodontal disease is diabetes mellitus.

In our study, the main values of the plaque index (QHI) which reflects the level of oral hygiene are presented in figure II.1.1.13, where one can observe: QHI control group = 3.3, QHI of the good metabolic control IDDM subjects = 3.4 and poorly metabolic controlled IDDM QHI = 3.6. Therefore, considering this assessment tool used to evaluate the thickness of plaque at the gingival margin, no significant differences between the subjects of the three groups were recorded into our study.

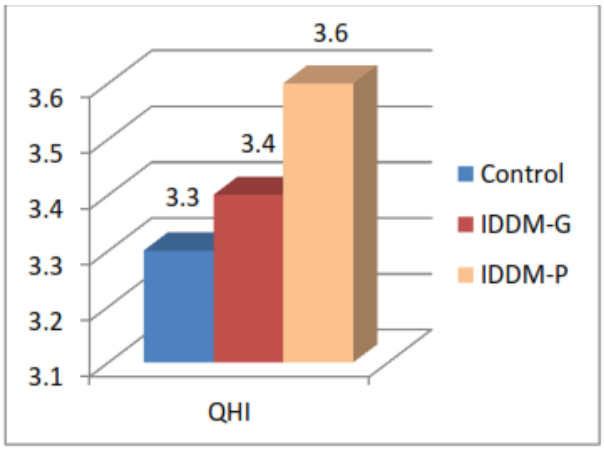

Figure II.1.1.13. *Levels of the plaque index (QHI) in Control group, good control of diabetes disease (IDDM –G) and poor metabolic control of the disease (IDDM-P)*

Hyperglycemia impairs overall cell function, as insulin is required for glucose to enter cells to provide a source of energy. It also declines PMN cell chemotaxis, phagocytosis and intracellular killing of bacteria. The ability of glycosylated hemoglobin to carry oxygen would be impaired, thereby reducing tissue oxygenation. Hyperglycemia induces blood flow abnormalities including increased blood viscosity, reduced erythrocyte deformability, and increased platelet aggregation, which further enhance tissue hypoxia, altogether triggering periodontal breakdown.

During clinical assessment, another clinical index reflecting periodontal homeostasis has been recorded, gingival index (GI) registering critical differences, with minimal inflammation (GI = 0.9) in subjects of the control group, by contrast to subjects with IDDM in which inflammation and bleeding was very important; with GI = 1.4 in subjects with good metabolic control of IDDM and GI = 1.8 in IDDM subjects with poorly metabolic control (Figure II.1.1.14), otherwise consistent with other results (Dakovick & Pavlovic, 2008).

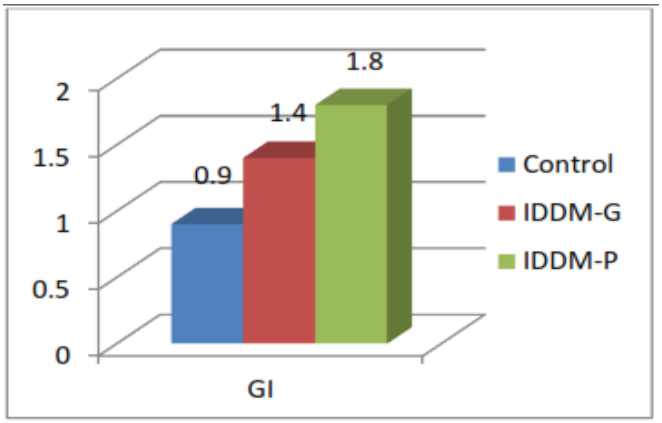

Figure II.1.1.14. *Levels of the gingival index (GI) in Control group, good control of diabetes disease (IDDM –G) and poor metabolic control of the disease (IDDM-P)*

As periodontal disease progresses, clinical attachment loss (CAL) occurs through the destruction of the periodontal ligament and its adjacent alveolar bone, subsequently leading to gingival recession and pathologic periodontal probing depth. he degree of CAL reflects the severity of CAL and can be used as an indicator to estimate the severity of periodontal disease. CAL, which measures the distance between the cement-enamel junction and the lowest point using a periodontal probe, is a criterion for the assessment of the severity of periodontal impairment.

Clinical attachment loss (CAL) was detected with levels between 1-2 mm in three patients with good control of the diabetic disease, higher values (CAL > 2 mm) being recorded in only two subjects with poorly controlled IDDM, all these patients aging between 12-16. The literature data upon this parameter are quite contradictory. Poor glycemic control in patients with diabetes has been also asociated with an increased risk of progressive loss of periodontal attachment and alveolar bone over time (Hanes et al., 2010) Although periodontal disease occurs primarily due to bacteria within the gingival crevice or the periodontal pocket, it may be affected indirectly by many other risk factors occurring changes in the vascular system, severity of inflammatory reactions and systemic immunological responses.

However, other studies failed to identify any significant relationship between glycemic control and periodontal status (Grover & Luthra, 2013), reporting exclusively gingivitis in children with DM, clinical attachment loss being rather absent. GCF volume measurement recorded more elevated mean values in diabetic children *versus* control (Figure II.1.1.14), the largest levels being recorded in children with poorly controlled metabolic disease (GCF volume = 1.02 ml), followed by 0.87 ml in the group of children with good metabolic control of diabetes and 0.46 ml in control group. According to Griffiths the volume of GCF is directly proportional to the stage of periodontal inflammation, which underscores its importance as a valuable assessment tool (Griffiths, 2003).

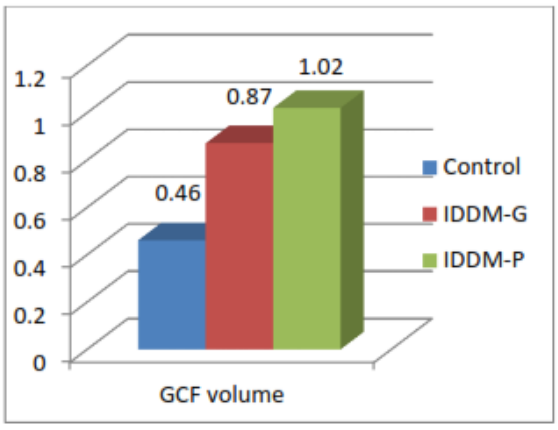

Figure II.1.1.15. *Levels of GCF volume (ml) in Control group, good control of diabetes disease (IDDM –G) and poor metabolic control of the disease (IDDM-P)*

In both groups, regardless of the glycemic control, the majority of the examined sites harbored dental plaque. Gingival bleeding was present at significantly higher degree

in the diabetic young population, with an evidence elevation in the older age group (3.13 ± 1.26 *vs.* 1.5 ± 0.82 in the 15-18 years group compared to 2.78 ± 0.78 *vs.* 1.23 ± 0.59 recorded among 11-14 years old patients).

Considering the third clinical indicator, no attachment loss was recorded in the prepuberal age, while clearly higher levels were correlated to the lower degree of metabolic control of the diabetes. Thus, various degrees of periodontal attachment level were registered between the studied groups in the 15-18 years subjects (1.03 ± 1.54 in non-diabetics and 2.56 ± 1.26 among poorly controlled diabetic teenagers).

II.1.1.4. Discussions

The association between DM and periodontal disease has been debated over decades, with conflicting conclusions. Most of the recent studies tend to support a higher prevalence and severity of periodontitis in diabetic adult patients, less literature data being available in what concerns insulin dependent diabetes upregulation of periodontal breakdown in children and adolescents. It has been shown that diabetes strongly influences the production of inflammatory mediators, cytokines and chemokines (Joo and Lee, 2007) resulting in abnormal immune inflammatory reaction and tissue injury in patients with periodontitis. Periodontal disease represents a group of alterations with episodic evolution that affects gingiva and could secondly alter the surrounding connective tissue.

Diabetes mellitus and periodontitis exist in a bidirectional cyclical relationship, with diabetes leading to oral disease, and periodontitis, in turn, exacerbating hyperglycemia. Periodontitis is recognized as the sixth major complication of diabetes, having increased prevalence and severity in patients with diabetes. Early diagnosis of diabetes in patients with periodontitis can lead to the prevention of major morbidity and mortality associated with the disease.

The main purpose of our study was to examine the interplay between the local and systemic cytokine profile, and immune-inflammatory mediated clinical response, in systemically healthy and insulin dependent diabetes mellitus young subjects. In order to achieve this goal, we used flow cytometric techniques to characterize the levels of some pro and anti inflammatory cytokines both in serum and gingival fluid. In addition, the study tempted to reflect the clinical changes in the oral health within children and adolescents with type 1 diabetes mellitus, to assess the rate of gingival inflammation accompanying the systemic disorder and to contribute to the incidence data on periodontal disease for groups of patients where factors attributable to aging are not confounding variables.

The investigation was carried out on 84 subjects age 6-18 years, divided into two main groups: The first group (diabetic group) consisted of 42 subjects with type 1 diabetes mellitus diagnosed with marginal chronic periodontitis (n=6), aggressive periodontitis (n=6) and gingivitis (n=30). The diabetic group was subdivided according to the level of metabolic control, into good control diabetes, glycosylated hemoglobin levels ≤7% (n=22), and poor control diabetes with glycosylated hemoglobin levels > 7% (n=20). In the second group (non-diabetes group=ND), there were 42 age-matched subjects who did not suffer from any systemic disease, most of them experiencing the mildest form of periodontal

breakdown, gingivitis (n=36), followed by marginal chronic periodontitis (n=4) and aggressive periodontitis (n=2).

Estimation of parameters related to distribution, diagnosis and age reveals the highest prevalence of gingivitis, the mildest form of plaque-induced inflammatory disease (85,7%), followed by breakdown of the superficial periodontal support (chronic superficial marginal periodontitis) (9,5%) and aggressive periodontitis (4.8%) in the non-diabetes group. Considering the diabetes group, there were different incidences of periodontal disease in the two subgroups: children and adolescents with good metabolic control (n=22) displayed generalized bacterial gingivitis in a proportion of 81% (n=18) and 19% aggressive periodontitis (n=4), while in the poorly controlled diabetes group, besides bacterial gingivitis (60%, n=12), 30% were diagnosed with chronic superficial marginal periodontitis (n=6) and 10% with aggressive periodontitis (n=2).

Thus, despite of some previous results that founded no significant correlation between gingival condition and glycosylated hemoglobin levels (De Pomereau et al., 1992), our data suggest that at young ages, there is a higher incidence and severity of periodontal breakdown in poorly controlled diabetes, where the incidence rate increases after puberty and continuously increases by age, with an overall elevation in resorption of the bone and epithelial attachment, and predisposition to infection.

Diabetes mellitus is a systemic disease with several major complications affecting both the quality and length of life. One of these complications is periodontal disease. Periodontal disease (periodontitis) is much more than a localized oral infection, recent data indicating that periodontitis may cause changes in systemic physiology. The interrelationships between periodontitis and diabetes provide an example of systemic disease predisposing to oral infection, and once that infection is established, the oral infection exacerbates systemic disease. The relationship between periodontitis and diabetes has been extensively investigated over the last years, but despite of the numerous scientific studies on the influence of periodontal treatments on glycemic control, there is limited knowledge on the impact of glycemic control upon periodontal status. Moreover, the impact of periodontal treatment on sugar metabolic control in diabetics has not been fully elucidated, the present chapter intending an outlining of the features that governs the interrelationship diabetes mellitus – periodontal disease, a discussion of the present scientific evidences, mainly focusing on clinic-biological research in juvenile groups of population.

The main pivotal mechanisms related to the etiology and pathogenesis of the diabetic complications include:

a. increased oxidative stress with excessive production of reactive oxygen and nitrogen species (Robertson and Harmon, 2006) and decreased antioxidants (Simmons, 2006);
b. the polyol pathway, resulting in toxic complications induced by sorbitol and
c. production of advanced glycosylation end products (AGEs) associated to impaired lipid metabolism.

These concepts propose that glucose binds, by non-enzymatic reaction, to proteins such as hemoglobin, collagen, or albumin, determining certain complications triggered by

the AGEs-released mediators. Diabetes complications, long time exclusively assigned to hyperglycemia can be equally determined by lipid metabolism impairment, characterized by serum LDL (low density lipoprotein), TG (triglycerides) and FA (fatty acids) level augmentation.

Lipid imbalances may be related to monocytes function disorder, monocytes being able to elicit suppression of growth factors production, therefore expressing an inflammatory phenotype (rather than a proliferative one), consecutive stimulation by the pathogenic bacteria endotoxin (lipopolysaccharide). Moreover, most of the evidences from the literature prove that higher levels of serum triglycerides induce stimulation of monocytes production of pro inflammatory interleukins on one hand, and of chemotactic and phagocytic abilities of neutrophils on the other hand (Iacopino, 2001).

Among the others cavities of the body, the oral cavity represents a distinctive ecosystem endowed with critical important biological functions, the fluids that bathes the mentioned ecosystem possessing an impressive number of components. Among the inflammatory disorders, periodontal disease-PD represents gram-negative anaerobic infections that involve tooth supporting tissues, the structures that form the periodontium (gingiva, alveolar ligament, root cementum, and alveolar bone). These alterations have mainly episodic evolution affecting first the gingiva and followed by possible secondary alteration of the surrounding connective tissue.

The most widely used classification was the American Association of Periodontology classification that distinguishes six categories: gingival disease, chronic periodontitis, aggressive periodontitis, periodontitis as manifestation of systemic disease, necrotizing periodontal disease, and periodontal abscess (Armitage, 1999). Actually, being no well-defined clinical criteria for the diagnosis, periodontal disease cannot be classified according only to the etiology, the designation periodontal disease including both reversible, soft form of inflammation, gingivitis, and irreversible, more extensive processes, periodontitis, tightly associated not only to the connective tissue of the tooth support destruction, but also accompanied by apical migration of the whole apparatus. It is one of the most widespread diseases in the world, the clinical importance of periodontal disease deriving partly from its very high prevalence, both in developed and developing countries. The main representative clinical manifestation of periodontal disease is the appearance of periodontal pockets, real favorable niche for microbiological colonization, relative facile to be revealed by clinical investigation with the periodontal probe and paraclinical X-ray imaging.

It is well known the fact that, although necessary in initiating the state of disease, bacteria represent insufficient criteria to determine its progression in the absence of an associated immune response. Also, despite the fact that the response of the host and environmental factors are important in manifesting the state of disease, nor gingivitis, neither periodontitis can onset in the absence of bacterial triggered mechanisms (Noda et al., 2007). The inflammatory reaction in the context of periodontal disease, initiated by the accumulation of bacterial plaque, starts in early childhood and reflects the special significance of the bacterial impact on the host, in a systemic context. At most children, the inflammatory process of the gum remains superficial – at the clinic stage of gingivitis, but

there are cases where the balance between the bacterial aggression and the host response is impaired, leading to destructive processes which induce attachment loss, and even lost of the teeth. Moreover, Armitage includes in his classification the pre puberal periodontitis, juvenile periodontitis and the quick progressing forms of manifestation of periodontal disease at children and teenagers, in the aggressive periodontitis class, because of the fast progression and severe impairment of periodontal tissues (Armitage, 2000).

This is why, tracking down the disorder as early as possible, is essential for an early establishment of a specific therapy, but especially for preventing the installing and evolution toward more severe forms of disease. On the other hand, the inconsistency between the aggression of periodontal destruction at child and teenager and the reduced quantity of biofilm (in some forms of tooth decay), determined some scientists to claim that the bacterial challenge represents an essential condition, although not sufficient in developing periodontitis, the decisive factor being actually, host susceptibility (Tabholz et al., 2010). Today, it is well known that both genetic and contracted factors are determinants of periodontitis presence, progression and severity in adults, Pihlstrom attributing to genetic causes almost half of the risk in developing a periodontal disease during life (and probable to be revealed even during childhood) (Pihlstrom et al., 2005).

Analysis of clinical parameters related to distribution, diagnosis and age highlights significant differences in the prevalence of severe periodontal breakdown between the two principal studied groups, with an 14.3% overall prevalence of chronic marginal periodontitis and aggressive periodontitis within systemically healthy individuals versus 28.5% in IDDM group.

Moreover, the same proportion was maintained when considering the two diabetes subgroups, almost two times more subjects with poor controlled diabetes experiencing severe periodontal injury (40%) compared to good metabolically balanced age-matched diabetic individuals (19%). Thus, despite that the oral health status data showed gingivitis as the main periodontal alter in both groups, there were significant differences among diabetic subpopulations (81% versus 60% within good and poor controlled diabetics, respectively). This was followed by a 3.1-fold increased incidence of chronic superficial periodontitis within the diabetics (30%) compared to non-diabetic group (9.5%), and almost twice more prevalent aggressive periodontitis in IDDM children and teenagers (19% *vs.* 10%).

Summarizing the results based on clinical diagnosis of periodontal injury related to age interval, the highest prevalence of gingivitis is specific for the prepuberal age, followed by an increase incidence of marginal superficial chronic periodontitis in puberal stage and forms of aggressive periodontitis during juvenile age, among all studied groups. Considering the two main population groups, gingivitis is the main periodontal alter among systemically healthy subjects, the associated systemic disease eliciting an increase in the incidence of more severe periodontal breakdown.

Periodontal homeostasis breakdown along systemic alteration of type 1 DM was also assessed through evaluation of clinical parameters (PI, PBI, CAL) correlated with age, duration and metabolic control of diabetes mellitus (HbA1c values). Statistically significant differences were recorded both, between the mean PI values corresponding to

the non-diabetes group and poorly controlled DM individuals, and between the two diabetes subgroups ($p<<0.05$, CI=95%). Moreover, taking into consideration the age intervals, comparative statistic studies of the oral hygiene parameter highlight the most significant differences among the juvenile age individuals, for all studied groups ($p<<0.05$).

As highlighted in Table II.1.1.2, papillary bleeding index in diabetic children and teenagers have significantly higher values, directly correlated to the age of systemic disease ($r=0.64$). Taking into consideration the distribution by age, the most important statistical difference is registered along pubertal period, pointing out a significant difference between the mean PBI values in ND patients (PBI=1.23) versus good controlled diabetic group (PBI=2.65) ($p=0.007$, $p<<0.05$). Furthermore, mean BPI significantly differs in patients with poorly controlled diabetes than the average values in patients with good controlled diabetes and ND ($p<<0.05$), this pattern of overall changes in inflammatory periodontal parameter's levels persisting across all age groups (prepubertal, pubertal, juvenile). These results can be explained by increased activity of collagenases and vascular changes within diabetes that increase gums bleeding and thickening of the small vessels basal membrane of the gingiva.

Distribution of CAL values indicated the most elevated (between 1.5 - 4 mm) and highest mean level (CAL = 1.053 mm), in the group of subjects with poor controlled diabetes (Table II.1.1.3), about 2.7 and 3.25 times higher than that of ND (CAL = 0.388 mm) and good metabolic controlled IDDM (CAL = 0.324 mm), respectively. Reffering to age, the highest mean loss of attachment characterized the 15-18 years old poorly controlled IDDM subjects (CAL=2.56mm), 2.4 more elevated than in ND (CAL=1.03 mm) and 3.2 times higher than in good metabolically controlled diabetics (CAL=0.79mm) (Table II.1.1.3). In prepuberal stage, almost no one can question the loss of attachment (explained both by anatomic and physiologic characteristics of this phase and the very short period in which teeth are maintained on the arch). In addition, the disease's evolution is insufficient to elicit real periodontal breakdown of chronic marginal periodontitis type, most commonly, loss of attachment being rather related to diabetes time course.

Furthermore, HbA1c values correlated with clinical parameters of oral status indicated that poorly controlled diabetes (HbA1c >7%) is associated with elevated bleeding index. Comparison of the parameters that indicate the degree of periodontal disruption (PBI and CAL) with age of onset of systemic disease revealed that age of diabetes and its metabolic control could be important determinant indicators to evaluate DM as a risk factor for periodontal breakdown within children and adolescents.

Determination of gingival crevicular fluid with parallel serum levels of soluble inflammatory mediators is highly relevant for studying children and teenager periodontitis within systemic context, since this consistent oral fluid, which bathes the periodontal pocket, derives from gingival capillary beds and contains resident and emigrating inflammatory cells. Systemically healthy patients with the mild form of periodontal disease recorded the lowest IL-1β gingival fluid level, a significant increase of this mediator being associated to IDDM group. Moreover, in ND patients there was a dose–response relationship between the severity of periodontitis and gingival crevicular fluid IL-1β levels

(two times higher mean values in systemically unaffected subjects with aggressive periodontitis), which suggested that periodontal disease may play a major role in elevating levels of this cytokine.

Our results reveal an overall pattern of most prominent variability among poorly controlled diabetic children and teenagers, regardless of periodontal breakdown degree (gingivitis or aggressive periodontitis). Data from the literature are somehow conflicting, certain results on adult population mentioning the lack of correlation between production of IL-1β related cytokine, and HbA_{1c} levels in patients with type 2 diabetes and periodontitis (Engebretson et al., 2007). Moreover, our data recorded significant positive correlation (Pearson test, r = 0.73) between IL-1β and glycosylated hemoglobin levels in diabetic young individuals, translated also into increased interleukin levels directly related to the reduction in the degree of metabolic control of the systemic disorder. Poor glycemic control is associated with elevated GCF IL-1beta. These data are consistent with the hypothesis that hyperglycemia contributes to a heightened inflammatory response, and suggests a mechanism to account for the association between poor glycemic control and periodontal destruction

IFN-γ is an inflammatory cytokine associated with inflammation, tissue destruction, bone resorption and specific elevated production of collagenases, serum and local determinations of this important regulator of immune inflammatory response revealing different levels in diabetic individuals, higher when associated to a good metabolic control and more specific periodontal breakdown. Moderate IFN-γ serum levels were recorded in diabetic population compared to ND, the high expression of gingival fluid cytokines in severe periodontal alteration of these patients being probably a marker of continuous Th1 response against microbiologic challenge, especially bacterial pathogens colonized in gingival tissue. This can be explained by alterations in the oral microenvironment caused by much higher amounts of glucose and urea in gingival fluid from DM individuals (data recorded by laboratory analysis of gingival fluid), that create a favorable environment for bacterial changes, with alteration of host immune response to periodontal pathogens, and suggests that Th1 mediated cytokine response may play a destructive role in the periodontium.

The present results indicate that microbiological overlapping involves considerable efforts of the body, resulting in significant elevation of IFN-γ, but not of IL-2 which was very low both, in blood and GCF diabetic individuals, suggesting the possible existence of a local factor that blocks the lymphocyte and macrophage secretion of this T lymphocytes factor of proliferation, mainly in diabetic patients with periodontal deterioration. The reasons for moderate IL-2 secretion are probably complex and may involve transcriptional or translational repression.

IL-10 registered decreased secretion in the diabetic population, both gingival fluid and serum values recording significantly higher differences between the two systemically affected subgroups (Figure II.1.1.6). Effective immune responses against pathogenic microbes depend on the balance between pro-and anti-inflammatory reactions. Interleukin-10 (IL-10) is essential in regulating this balance and has gained interest recently as a modulator of the response to infection at the Janus Kinase-Signal Transducers and

Activators of Transcription (JAK-STAT) signaling axis of host responses (Carey et al., 2012). It is thus possible that reduction in IL-10 secretion within juvenile diabetic population could play an important role in the shift of the oral tissue differentiation toward periodontal injuries. Interleukin-10 is a prototypic anti-inflammatory cytokine generated in response to a variety of pathogens, that exhibit a central role in limiting host immune response to pathogens, thereby preventing breakdown of the host and maintaining normal tissue homeostasis, dysregulation of IL-10 being related to enhanced immunopathology in response to infection as well as augmented risk for development of bulk disorders (Hutchins et al., 2013). IL-10-mediated anti-inflammatory response represents an essential homeostatic mechanism responsible for regulating the degree and duration of inflammation. It also declines secretion of inflammatory cytokines, including TNF-α, IL-1, IL-2, interferon, and granulocyte-monocyte colony-stimulating factor (GM-CSF), and several chemokines (like IL-8), as well (Duell et al., 2012).

The prevalence of type 1 DM exhibits a wide range, especially in Europe, the children being extensively and continuously affected. Patients with diabetes have increased incidence and severity of periodontal disease. Poor glycemic and metabolic control has been consistently associated with periodontal disease severity (Foia et al., 2012).

There is large evidence that pre-diabetes is a risk factor for the development of periodontitis. But the mechanisms underlying such relationship are far of being revealed. Toll-like receptors, through NK-B activation, are involved in periodontitis pathogenesis and, thus, could be involved in pre-diabetic-enhanced inflammatory processes. The development of periodontal inflammation in rats with induced pre-diabetes is triggered through expression of toll-like receptors of type 2 and 4, and further activation of cascades involving NK-κB (Meurman, 2018). Hyperglycemia is inducing wide alterations of all metabolisms as well as of innate immune system functioning and reactivity. There are large evidences suggesting the deep involvement of local enhanced inflammatory responses in the alterations of periodontal tissues as a result of diabetes aggression. The released cytokines, matrix metalloproteinases, free radicals of oxygen are enhanced, having as result an aggressive local inflammatory response with the subsequent destruction of gingival tissues.

Toll-like receptors (TLR) expression and functioning is also altered as a clear result of accumulating advanced glycation end-products, these ones altering all the immune responses, local or systemic ones. RANKL/osteoprotegerin axis is also a target for hyperglycemia during the non-catalytic attack of advanced glycation end-products on all tissues (Meurman, 2018). Local migrated leukocytes induce high pressures on endothelial cells through an enhanced expression of integrins and selectins as well as an augmentation of IL-1, IL-6 and TNF-α secretion.

When compared to healthy periodontal subjects, the patients with periodontitis present different amounts of released pro- or anti-inflammatory mediators throughout their gingival tissues and gingival crevicular fluid. Thus, IL-1α, IL-1β, IL-6, TNF-α and IFN-γ are increased in different degrees in various stages of periodontitis in adult patients. On the other side, the concentrations of IL-4 are consistently declined in all periodontal and gingival impairment stages. All these above-mentioned changes develop the basis for

progressive destruction observed in periodontal diseases as a result of enhancement of inflammatory reactions, local or systemic ones.

II.1.1.5. Conclusions

Our studies showed that DM modulates GCF expression of Il-1β, IL-2, IL-4, IL-5, IL-10, TNF and IFN-γ in patients with impaired periodontal territories. Very probably this is the result of immune system cells sensitization by endogenous ligands and bacterial products through various receptors, some of them recognized as important mediators of immune responses in inflammatory diseases. Thus, the present study clearly reinforces that children and adolescents are susceptible to destructive forms of periodontal disease, especially when the etiologic external factors (microbial flora) are associated with host-related systemic impairment, such as insulin dependent diabetes. In summary, our data support the notion that systemic alteration of IDDM type is associated with distinct patterns of GCF cytokine expression.

Poor controlled young diabetic subjects were characterized by local higher IL-1β and decreased IL-10 and IFN-γ amounts, compared to systemically healthy subjects, suggesting that an imbalance between pro- and anti-inflammatory cytokines is associated with the possible switch of the biofilm-modulated periodontal status toward more specific breakdown. IL-1β, IL-10 and IFN-γ might be involved in controlling the inflammatory process at periodontally healthy and diseased sites, the present data indicating that the interactions appeared to be different in subjects that were systemically healthy when compared with IDDM subjects.

Moreover, the metabolic equilibrium of the systemic disease is significantly related to the gramm negative species mediated cytokine translocation from the periodontal space into the circulation. Further studies of candidate biomarkers and of inflammatory shifts will be necessary to confirm these observations. The differences of cytokines secretions between groups of diabetic patients with or without periodontal alteration are more apparent in serum. The diabetic body interferences with microbiological microenvironment imply serious efforts of the first one, resulting in a significant local and systemic secretion of chemokines and interleukins which are also enhancing alterations induced by the hyperactivity of immune system. In these cases, periodontitis could be the evolution result of hyperglycemia attack on gingival tissues.

Our results indicate the existence of important relationship between the presence of metabolic impairment of diabetes nature in children and adolescents and changes in the marginal periodontium of these subjects, important correlations being established particularly with indicators of inflammation (gingival index and gingival crevicular volume). Clinical attachment loss was recorded only in a few casess of diabetic children aged between 12-16 years, enrolled in the survey. In summary, as the literature data considerations upon IDDM child and adolescent impairment of the periodontium are conflicting and scanty, our results ar consistent with some of them but in disagreement with others.

Despite some conflicting data in the literature, our findings support the observation of a positive relationship between the bleeding index and the clinical attachment loss and

immune alterations in these patients, with subsequent increase in immune inflammatory mediator production that might be ultimately responsible for the damage observed in the periodontium, and systemically as well. Periodontal alter is more prevalent and severe in type 1 diabetes mellitus young subjects. A greater periodontal inflammatory tendency corresponded to those individuals with poorer metabolic control, while metabolic disbalances associated greater periodontal attachment loss, diabetic children and teenagers being more vulnerable to periodontal breakdown.

II.1.2. Evaluation of the periodontal modifications, sociodemographic and behavioral response associated with intellectual and developmental disabilities in young subjects with Down syndrome

A second direction of research included the exploration of periodontal changes in children with mental challenges such as Down Syndrome. Through the nature of our profession, I have come in contact with this pathology in a category of children with special needs commited to both empathy and professionalism from practitioners in the field of dental medicine. Foremost, it is critical that medical professionals provide accurate and current information to parents facing a difficult diagnosis in a supportive and compassionate manner. The way the diagnosis is provided the parents and how they are supported by medical professionals seems to be as important as the diagnosis itself. That is why, during my researche I have either focused on assessing the periodontal status as well as the evaluation of the biochemical changes of the gingival fluid associated to the critical intellectual and developmental disabilities.

II.1.2.1. STATE OF THE ART

Down syndrome (DS), also known as trisomy 21 is the most frequent chromosomal abnormality, which characteristically has significant cognitive disability and neurologic deficiencies. It affects 1/700 to 1/1000 live births (Perluigi et al., 2014).

Down syndrome individuals present anatomical abnormalities, mental and orofacial problems with a critical impact in quality of life (Shyama et al., 2003). Furthermore, DS patients are more susceptible to infections, including an increased prevalence of periodontal diseases, almost 100% under the age of 30 years (Meyle & Gonzales, 2001). Periodontal alter in these patients is severe, generalized, with rapid progression and classified as a manifestation of systemic diseases associated with genetic disorders by American Academy of Periodontolgy (Armitage, 1999).

Poor oral hygiene *per se* may not explain severe and generalized periodontal destruction observed in DS patients. This condition is also associated with impairment of immunological system (Cavalcante et al., 2012). DS patients dispaly mild to moderately reduced T and B cell counts, absence of normal lymphocyte expansion in infancy, suboptimal antibody responses to immunizations, declined immunoglobulin A in their saliva or neutrophil chemotaxis, as well (Ram & Chinen, 2011).

Current data revealed elevated level of TNF-α and IFN-γ in children with DS, these inflammatory cytokines present biological effects in the body and important regulatory roles in immune responses. Some research demonstrated altered expression of immune-related genes in children with DS, highlighting molecular mechanisms involved in DS pathology (Zampieri et al., 2014). Moreover, some local disorders are related to the development of early periodontal breakdown, such as poor occlusal correlation, high frenum insertion or early mucogingival problems. In addition to periodontal therapy, these subjects must apprehend attention and management of dental caries, malocclusion and obstructive sleep apnea (Meurman, 2018).

There is a growing interest in the contribution of the immune system in the development and progression of Down syndrome disorders. A study from 2017 evaluated the coenzyme Q10 and selected pro-inflammatory markers such as interleukin 6 and tumor necrosis factor in children with Down syndrome. Forty-three young Down syndrome children and forty-three controls were included over a period of eight months, and found that, compared with the control group, the DS subjects recorded significant augmentation of IL-6 and TNF ($p = 0.002$), while coenzyme Q10 was greatly diminished ($p = 0.002$). Moreover, fasting blood glucose and body mass index were increased in a consistent degree. There was a significantly positive correlation between coenzyme Q10 and intelligence quotient levels, as well as between the two investigated cytokines (Tiano & Busciglio, 2011).

Severe blockage in the brain of patients with an extra chromosome 21 could be responsible for cognitive deficits noticed throughout their lives (Martínez-Cué et al., 2014).

Oxidative stress is known to have a substantial role in the pathology as well, because of genetic and epigenetic factors, which suggests that oxidative imbalance contributes to the clinical manifestations in Down syndrome (Tiano et al., 2012). In DS patients, the oxidative damage has a critical role in the neurodegenerative events. Some studies have revealed that trisomy 21-related boost of oxidative stress might be involved in different aspects of DS phenotypes (Perluigi & Butterfield, 2012). Coenzyme Q10 (CoQ10) behave as a reactive oxygen species (ROS) scavenger, very probably by activating oxidative damage repair enzymes and with a role in gene expression regulation. Nevertheless, it might act as a DNA repair mechanisms modulator. The correlation of CoQ10 levels to IQ scores in DS patients implies the CoQ10 effect on neurodevelopment process, that may be due to its protective role against nuclear DNA damage, in addition to its redox operation plan (Tiano et al., 2012).

In other studies, some inflammatory biomarkers and oxidative stress mechanisms in DS children have been evaluated and compared to controls, all patients recording critical cutback in plasma CoQ10, a potent endogenous antioxidant, which in turn, might be an important factor associated with oxidative imbalance in children with trisomy 21. The authors found significant decrease of CoQ10 in DS patients (Miles et al., 2007).

The effect of CoQ10 has been studied in some neurological disorders where mitochondrial dysfunction was detected. Studies comparing data on oxidative DNA damage and systemic oxidative stress parameters in CoQ10-treated DS patients concluded

that CoQ10 does not simply work as an ROS scavenger. This is because of the absence of a measurable plasma antioxidant response, which could be masked by the hyperuricemia usually found in DS, together with unchanged DNA levels (Tiano, & Busciglio, 2011). Important outcomes have been issued following coenzyme Q10 administration to DS patients, in an attempt to counteract the oxidative imbalance present due to its secondary deficiency (Littarru & Tiano, 2010).

Individuals with DS are more prone to infections and autoimmune disorders. Ineffective immune responses in DS lead to recurrent viral/bacterial infections and contribute to the development of various pathophysiological symptoms, including cognitive impairment (Rostami et al., 2012).

The dysfunction of the immune system in DS has been attributed to reduced number of B-lymphocytes, T-cell subset modifications, as well as changes in the levels of anti- and pro-inflammatory cytokines. Tumor necrosis factor (TNF) and interleukin 6 (IL-6) have been involved as key components of immune and also inflammatory processes. An improved and better understanding of the relationship between these different elements may help in the discovery of new approaches to ameliorate the progression of dementia in trisomy 21 patients (Rodrigues et al., 2014).

It has been postulated that a triplicated chromosome 21 causes a 50% increase in the expression of trisomic genes as a primary dosage effect, which translates directly into biochemical abnormalities (Abdel-Salam et al., 2013). A study by Tiano et al., concluded that lymphocyte and platelet CoQ10 content were significantly lower in DS patients, a fact which probably underlie oxidative imbalance at the cellular level (Tiano et al., 2008). The CoQ10 redox mechanism is possibly related to maintaining the mitochondrial homeostasis and prevention of free radical production.

High rates of overweight and obesity among children worldwide and the range of health problems associated with them from psychosocial to adverse metabolic findings warrant development of a number of action plans and setting of global targets for the prevention of obesity in children and adolescents. New preventive strategies, highlighting the important role of physical activity and nutrition education, are necessary. High body mass index - BMI is associated with a specific pattern of low-grade immune activation (Magrone & Jirillo, 2015).

Gardiner delineated that although intellectual disability in DS can be only mild delay, the most frequently reported IQ is in the range of 40-50 (mild to moderate delay) (Gardiner et al., 2014). Some reports claimed that the IQ score of the DS patients registered moderate mental disability in 31% and mild mental disability for 52% of their patients (Shukla et al., 2014). Data from literature point out that subjects with DS have an increased susceptibility to infections and autoimmune disorders, which are the main causes of mortality and morbidity observed due to this genetic alteration. The immune system dysfunction in DS has been associated to B lymphocyte decreased number, T-cell subset modifications as well as anti and pro-inflammatory cytokine level adjustments (Verstegen et al., 2010).

However, the molecular mechanisms leading to immune defects and the contribution of these alterations to the increased risk of infections have not been fully

elucidated (Xu et al., 2013). Extracellular adenine nucleotides and nucleosides such as ATP and adenosine have been recognized as key components of immune and inflammatory processes (Bours et al., 2016). ATP, acting through specific cell surface purinergic receptors, is involved in pro-inflammatory actions such as lymphocyte stimulation and proliferation, as well as cytokine release, including IL-2, IFN-γ, IL-1β and TNF-α. In addition, ATP induces secretion of cytokines like IL-2, IFN-γ, IL-1β, and TNF-α from activated lymphocytes and macrophages (Langston et al., 2003).

Alternatively, adenosine, a purine nucleoside and product of ATP hydrolysis exerts potent anti-inflammatory and immunosuppressive actions by inhibiting both, proliferation of T cells and secretion of pro-inflammatory cytokines, such as TNF-α and IFN-γ (Hasko et al., 2008). Extracellular ATP and adenosine levels are regulated by cell surface ectoenzymes, such as ectonucleoside triphosphate diphosphohydrolase (NTPDase) and adenosine deaminase (ADA). NTPDase1 (CD39) is involved in the breakdown of ATP and ADP to AMP which is hydrolysed by ecto-5′-nucleotidase to adenosine (ZebischM et al., 2013). Adenosine desaminase is considered a key enzyme in purine metabolism, catalyzing the irreversible deamination of adenosine to inosine, thus regulating extracellular adenosine availability (Phillis et al., 1991).

Since NTPDase1 and ADA activities potentially modulate extracellular levels of pro-inflammatory ATP and anti-inflammatory adenosine, the role of these enzymes has been evaluated in the pathogenesis of immune and inflammatory diseases. Indeed, the activities of these enzymes have been altered in such conditions, indicating the crucial role of NTPDase and ADA in the regulation of immunologic responses (Spanevello et al., 2010)

Acetylcholinesterase (AChE) is another enzyme involved in immune functions. This enzyme is expressed in both T and B lymphocytes and promotes the hydrolysis of the acetylcholine (ACh) to choline and acetate (Kawashima & Fujii, 2003). ACh is known to promote anti-inflammatory actions by suppressing the production of pro-inflammatory cytokines (Reardon et al., 2013). Thus, AChE emerges as a potential contributor in the pathways controlling inflammatory and immune responses mediated by muscarinic and nicotinic receptors (Nizri et al., 2013).

Alterations in the number and function of lymphocyte subsets have been correlated with the incidence of infections and autoimmune diseases in DS (Aburawi et al, 2012). Attenuation of anti-inflammatory and increase of pro-inflammatory mediators were shown in serum from DS individuals (Tanaka et al., 2012). Data from literature has demonstrated an increase of IFN-γ, TNF-α and IL-6 levels in adolescents with DS, Trotta et al. highlighting that mononuclear cells from peripheral blood of adults with DS in culture released more IFN-γ and TNF-α when compared to controls. Cytokine release may contribute to the disruption of an adaptative immune response development (Trotta et al., 2011). IL-1β and IL-6 are considered key orchestrators of immune and inflammatory responses. IL-10 is the most important anti-inflammatory cytokine of immune system by inhibiting the release of Th1 pro inflammatory cytokines, such as IL-2 and IFN-γ (Opal et al., 2000). It has been established that purinergic signaling contributes to the regulation of inflammatory and immune responses (Jacob et al, 2013).

Previous studies have demonstrated that the activities of a number of purine metabolizing enzymes are altered in lymphocytes and erythrocytes from DS subjects suggesting that purinergic signaling is altered in this genetic condition (Puukka et al., 1982). Extracellular ATP is involved in the pro-inflammatory function, hydrolysis of ATP by NTPDase1 playing probably a crucial role in immune suppression as it removes pro-inflammatory ATP and generates immunosuppressive adenosine (Parodi et al, 2013).

Apart from these genetic variations DS is associated with increased platelet aggregation, which can be an additional source of extracellular ADP as well. Hence, the augmentation of ADP hydrolysis observed in lymphocytes from DS subjects may contribute to adenosine overproduction. These alterations may represent an important compensatory mechanism to decrease inflammation and immune response in DS; ADA activity is increased in lymphocytes and erythrocytes from DS subjects, however, the origin of ADA in serum and the mechanism by which serum ADA activity is elevated have not been fully elucidated (Pourshari et al., 2009).

The purine nucleoside adenosine plays a crucial role in the regulation of inflammatory and immune responses by inhibiting lymphocyte activation and decreasing both Th1 and Th2 cytokine secretions through A2A receptor activation. In line with this, it is important to note that in serum of DS subjects the up-regulation of ADA activity may degrade adenosine, a molecule with immunosuppressive and anti-inflammatory actions and thus contribute to the high pro-inflammatory cytokine status (Haskó et al., 2013). Studies have demonstrated that the activation of the nicotinic receptors in macrophages reduces significantly the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, whereas the production of anti-inflammatory cytokines, such as IL-10, is not affected (Wang, 2003).

By the AChE activity blockade, it was possible to decline the levels of TNF-α and IL-1β in lymphocyte culture. In this way, inhibitors of AChE reduce lymphocyte proliferation and the secretion of pro-inflammatory cytokines and may elicit a reduction of inflammation by increasing the Ach concentration in the extracellular space (Nizri et al., 2013).

Changes in lymphocyte subpopulations such as an increase in T lymphocytes and a decrease of B lymphocytes have also been reported. These individuals are likely to possess a compensatory mechanism, such as an increase in IL-7 and IL-15, which have been reported as inductors of T cell proliferation and survival (Bloemers et al., 2011). Acoording to Hamertone, Down syndrome is characterized by the whole chromosomal aneuploidy in about 95% of cases. The remaining 5% is in the form of translocations and mosaics (Hamertone et al., 1961).

The data provided by National Down Syndrome Society in 2015 described more than 400.000 individuals with DS living in the United States. Still, life expectancy for DS patients increased dramatically in recent decades, from 25 years in 1983 to 60 years today.

Some data from a systematic review reported that patients with intellectual disabilities displayed poorer oral hygiene and more severe periodontal destruction than control patients (Anders & Davis, 2010). Involvement of family members, caregivers and institutional attendants are essential components in the periodontal treatment or prevention

programs. Cognitive deficiencies and reduced manual capacity to perform satisfactory dental hygiene should encourage more participation of family members/caregivers with this responsibility. Both DS patients and their caregivers should receive oral hygiene instruction. According to some researches, scaling and root planing as a primary therapy should be initiated early and with higher frequency for patients with such disabilities (Frydman & Nowzari, 2012).

Systematic review of Anders & Davis reported that impaired physical coordination and cognitive skills limit the ability of DS patients to independently perform sequential tasks such as daily tooth brushing. Thus, oral hygiene procedures are dependent of knowledge, attitude and supervision of a responsible person. However, many caregivers receive minimal training to assist DS patients in oral hygiene care. Their review described relevant information about oral health of patients with intellectual disabilities and reinforced the need for further research. In present, the strategies should target the increase of patient acceptance of routine periodontal and restorative dental care, and to minimize the need for this care with effective preventive procedures, dental health providing a huge impact on social acceptance and life quality (Anders & Davis, 2010).

Absence of proper supervision and negative attitudes toward dental health by the caregiver has been cited as obstacles to good oral health (Patrick et al., 2006). Teachers and institutional attendants should be prepared to early introduce disabled school-age children with effective methods to improve dental health. With this goal, settle down education programs for teachers, use of alternative materials and methods and again inclusion of the family and caregivers in dental health programs are essential. Professional local treatment and maintenance program associated with a rigorous home oral hygiene regimen are the key elements to assure an effective control of the disease in patients with special needs (Zaldivar-Chiapa et al., 2005).

However, no significant improvement in clinical and microbiological parameters after a single session of scaling and root planing and oral hygiene instructions could be observed (Hanookai et al., 2000). Data from other authors claimed that a frequent recall program could overcome the problems of poor hygiene in Down syndrome patients (Sakellari et al., 2001). Professional dental approaches are effective for reduce probing depth, plaque and bleeding indexes, but are impractical to be performed daily. Therefore, depending on periodontal condition, physical coordination, cognitive skills and participation of parents/caregivers, ideal frequency of assistance must be defined.

Among different chemical agents, chlorhexidine demonstrated reduction in plaque bacteria by up to 62% (Stabholz et al., 1991). Despite that many authors claimed that chlorhexidine mouthwash would be considered the gold standard for chemical plaque control, others reported that locally delivered chlorhexidine exhibit humble action on non-surgical periodontal therapy (Bonito et al., 2005). Thus, the role of single use of chlorhexidine mouthwash in the mentally disabled subjects to reduce plaque adequately is equivocal.

A favourable association between mechanical and chemical control of the dental biofilm in DS patients would be of potential interest, as some data revealed a positive impact on plaque and gingival inflammation levels in DS with poor oral hygiene through

association of a twice-daily chlorhexidine mouth rinse and use of gel for tooth brushing, in place of a regular dentifrice (Cheng et al., 2008). Nevertheless, considering positive outcomes of chlorhexidine, this could offer an effective preventive and therapeutic regimen for individuals with such disabilities (Freedman et al., 2011). Extensive clinical trials about preventive and periodontal treatment in DS patients are imposed, including antimicrobial agents and other adjuvant treatments.

My contributions to this research direction heve been included in the papers:

1. **Toma Vasilica**, Goriuc A, Cioloca D, Kozma A, Iordache C, Topoliceanu C, Filip F, Gamen A, Zegan G. Evaluation of periodontal status in a group of children affected by Down Syndrome. *Romanian Journal of Medical and Dental Education*, 2019;8(3): 28-34.
2. **Toma Vasilica**, Maxim A, Balan A, Gheban D, Rotaru DC, Filip F, Foia L. Periodontal aspects in children and adolescents with Down Syndrome. *Romanian Journal of Oral Rehabilitation*, 2009; 1(4): 35-42.
3. Balan A, Maxim A, Pasareanu M, **Toma Vasilica**, Balcos C, Barlean L. Socio-epidemiological aspects of odonto-periodontal pathology in the young adult between decades and actuality. *International Journal of Medical Dentistry*, 2012; 16(3): 167-171.

II.1.2.2. MATERIALS AND METHODS

We have conducted a study evaluating the periodontal status in a group of children (n = 24) with DS, aged 6-18 years from the Neuropsychiatry-Genetic Disease office of "St. Maria" Ambulatory Hospital, Iaşi, and a control group children (n = 24) without systemic diseases, who addressed for the dental treatment in the Clinic of Infant Dentistry in Iaşi.

The evaluation of the periodontal status was performed in the groups of children and adolescents through the calculation of the following indexes of diagnostic for periodontal disease:

- *The Quiqley and Hein coloured bacterial plaque index*
- *The intensity of inflammation***,** appreciated through the intensity of *papillary bleeding index* (PBI index) (Saxen and Muhlemann).
- *The level of attachment loss* **(**CAL) evaluated through periodontal probing and radiological exam.

All the evaluations of clinical indexes were performed at the level of Ramfjord teeth, mesial sites. The investigation of plaque deposits has been made through coloured plaque indexes Quigley Hein, as follows:

QH =	The sum of values for every tooth of B and L surfaces
	The total number of examined surfaces

The Rx exam, useful in appreciating the importance of attachment loss was performed on retro-dento-alveolar cliches in isometric and orthoradial incidence or through ortopantomography (OPT). Comparisons were made between the two groups for every calculated index, the values for each patient were calculated together for creating the average of the group, using a common ground established as standard of correction for the error of unequal variances. With this protocol, the research was centered upon the patients and not on the number of sites.

Experimental methods: Measurements of intracellular enzyme such as AST (spectrophotometry) and proinflammatory cytokine IL-1β (ELISA) were performed within GCF samples from the two groups (DS and control), each comprising 24 children and adolescents, aged between 6-18 years with different forms of periodontal alteration.

The clinical examination included periodontal probing of every site of all deciduous and permanent teeth and was carried out in the dentist's office, using individual and autoclaved instruments (according to biosafety requirements).

For gingival fluid AST determination, we used spectrophotometric method, a Hewlett-Packard spectrophotometer and the INIFINITY® AST test (Sigma), according to the manufacturer's protocol. The obtained data were directly expressed by the soft in U/l AST. For IL-1β determination an ELISA method was used, including an ELISA-plaque reader and a "Human Interleukinn-1β (hIL-1β)" ELISA kit. The IL-1β sampling was achieved by filter paper strips introduced for 30" in the mesial sites of the central incisive, premolars and permanent molars of the subjects. The IL-1β values of these subjects were compared in each group among the different forms of periodontal impairment.

It was performed the comparison of IL-1β values from the patients with systemic disease (DS) with the IL-1β values from the GCF of the control group also affected by different forms of periodontal alteration. Comparisons were also made between the values of IL-1β upon dental patterns in the studied groups, using a common background estimated as a correcting standard of error for unequal variances.

The statistic differences between the averages of the interest values according to groups were tested, using the One-Way ANOVA test completed by the Kruskal-Wallis test (or Mann-Whitney/Wilcoxon) for the level of IL-1β and AST from the GCF, age and clinical values. The statistically significant differences were considered for the level of signification (p) lower than 0.05, according to a level of confidence of 95%.

II.1.2.3. RESULTS

- ***The periodontal status evaluation in children affected by Down syndrome***

For the investigation of patients with Down syndrome from the point of view of clinical indexes, their values were compared with those recorded in the control group. In the analysis of each clinical index, the statistical indicators that correctly describe the characteristics of the batch were compared, comparing with the values of the indicators corresponding to the control group.

Thus, the QHI index recorded an average of 4.162 in the group of children with DS *vs* 3.495 in the control group (Figure II.1.2.1).

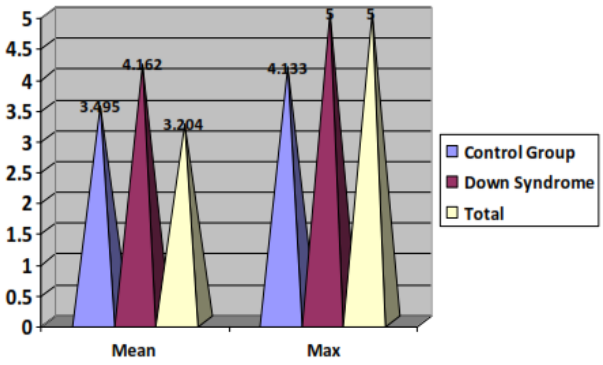

Figure II.1.2.1. *The mean values of the bacterial plaque index (QHI) in the control group and the group of patients with Down syndrome*

Although the values of the plaque index (QHI) (figure II.1.2.1) have increased, solely, microbial flora can not explain / justify the aggressiveness of periodontal disease in DS children and a number of responsible factors increased susceptibility to oral (generally) and periodontal (especially) infections in DS subjects.

The papillary bleeding index had an average value of 3.181, two-fold higher than on the control group - 1.562 (Figure II.1.2.2).

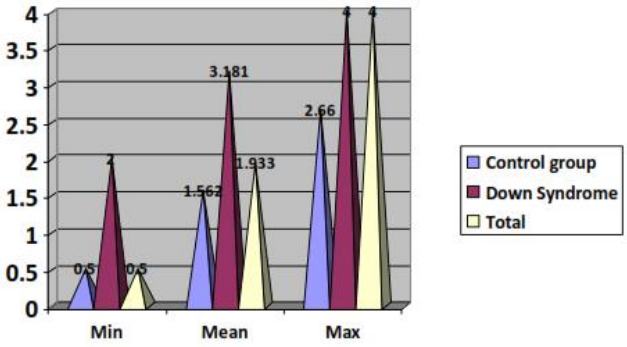

Figure II.1.2.2. *Average IBP scores in the control group and group of patients with Down syndrome*

Significant gingival bleeding in children with Down syndrome, explained by important bacterial plaque deposits, lack of oro-dental hygiene in these subjects, reached peak values in some cases, respectively PBI = 4 ("drop" bleeding). Also in the argumentation of this situation, other statements from the literature can be taken into account, such as: alter chemotaxis and intracellular degradation of neutrophils (Morgan et al., 2007), or the compromised immune system of DS children, the basis for the development of aggressive forms of periodontal impairment similar to localized aggressive periodontitis.

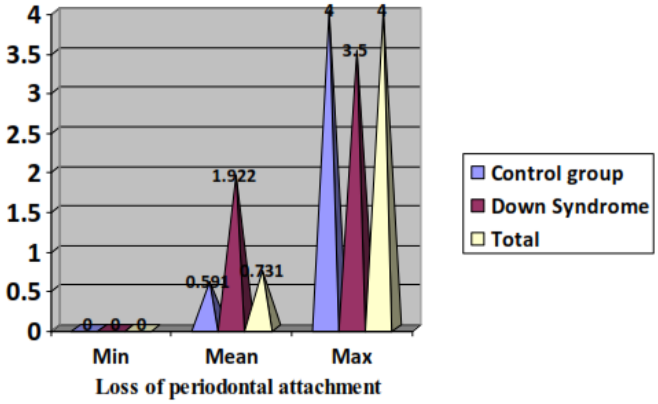

Figure II.1.2.3. *The mean values of the clinical attachment loss in the control group and the group of patients with Down syndrome*

As depicted in the above figure (Figure II.1.2.3), there is an increase of 3.25 times of the average value of the CAL index in the Down syndrome group compared to the control group (1.922 *vs.* 0.591)

Regarding the loss of periodontal clinic attachment (CAL), significantly increased values, even 4 mm, were recorded in the DS group compared to the children of the control group in which for the majority, CAL = 0. These changes might be trigerred by augmentation in prostaglandins, especially PGE2 values in GCF (Luchian et al., 2016) and other changes such as elevated activity of metalloproteinases such (MMP8 and MMP9) in polymorphonuclear (PMN) neutrophils and gingival fibroblasts (Surlin et al., 2012).

- ***Statistical evaluation of periodontal parameters in children and adolescents with Down syndrome***

The bacterial plaque index

For evaluating the patients with Down syndrome from the point of view of the clinical indexes, their values were compared to the values recorded in the control group. In the analysis of every clinical index the statistical indicators which describe correctly the characteristics of the group were calculated, comparing them at the same time with the values of the respective indicators of the control group.

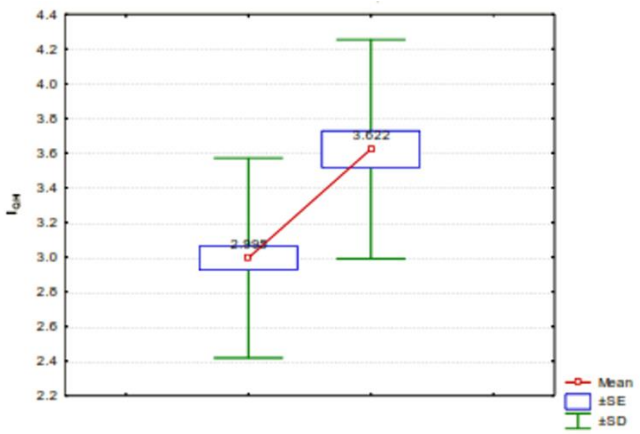

Figure II.1.2.4. *The mean values of plaque index in the control group and DS group*

Clinical Attachment loss (CAL)

In the figure II.1.2.5, one can notice an increase of 3.6 times higher of the average value for the CAL index in the Down group compared to the control group can be noticed.

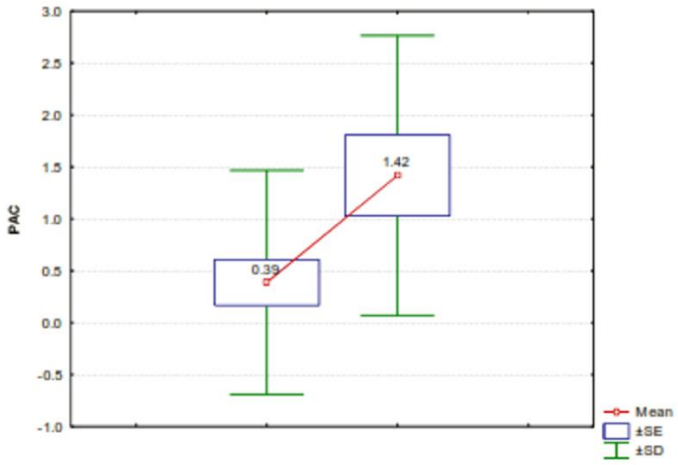

Figure II.1.2.5. *The mean values of CAL in the control group and DS group*

Papillary bleeding index

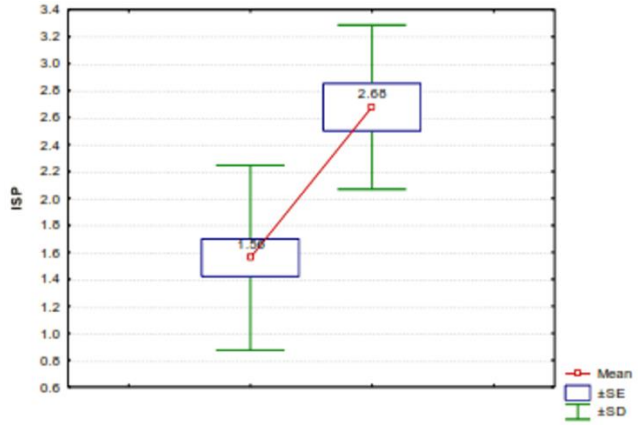

Figure II.1.2.6. *The mean values of papillary bleeding index in the control and DS group*

Corroborating the clinical exam with the values of the calculated clinical indexes (QHI, PBI, CAL) and with the radiological exam, we established the periodontal diagnosis for every patient. The evaluation of the parameters connected with the repartition depending on diagnostic and systemic condition showed the following:

Control group

The highest weight belongs to the microbial inflammatory diseases induced by bacterial plaque, gingivitis respectively (87.50%), followed by the superficial alteration of the sustaining periodontium (chronic superficial periodontitis) (8.33%), and aggressive periodontitis (4,17%) (Figure II.1.2.7).

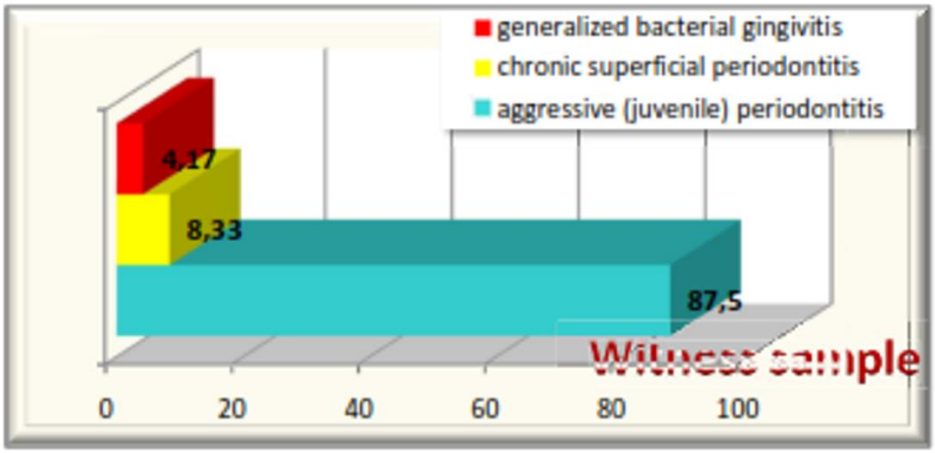

Figure II.1.2.7. *The incidence of cases depending on the periodontal diagnosis, in the control group*

The group of patients with Down Syndrome

Although the data from the literature show that in the patients with Down Syndrome aggressive forms of periodontitis are encountered (Shyama et al., 2003) in a proportion of 90% (Dow 1951) and between 90-100 % (Cohen et al., 1961), in our study, in the group of young subjects with Down syndrome, the bulk is represented by chronic superficial periodontitis (66.67%), followed by gingivitis (33.33%), the aggressive forms of disease being absent (Figure II.1.2.8).

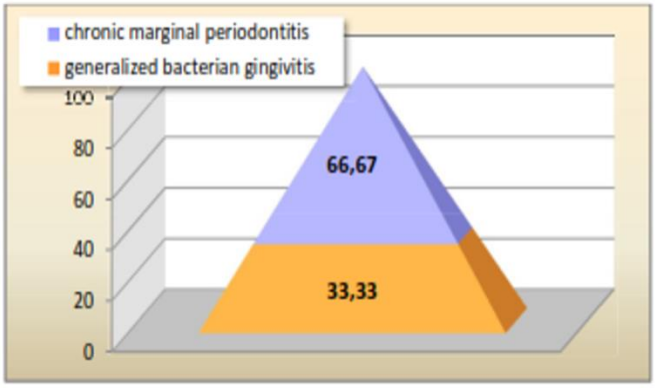

Figure II.1.2.8. *The repartition of cases according to the diagnosis in the DS group*

- ***Assessment of the periodontal status in Down syndrome individuals compared to control group***

In the Table II.1.2.2.1. are described the clinical periodontal parameters of the studied population, by age subgroup (prepuberal, puberal and juvenile).

Table II.1.2.1. *Clinical periodontal characteristics of the studied population; PI – plaque index, PBI – papillary bleeding index, CAL – clinical attachment loss.*

Clinical parameter	*Age*	*Control*	*Down Syndrome*
PI	6 – 10 years	3,17 ± 0,5	**3,66 ± 0,64 (p = 0,2116)**
	11 – 14 years	2,73 ± 0,44	**3,56 ± 0,78 (p = 0,00006)**
	15 – 18 years	3,03 ± 0,66	**3,66 ± 0,29 (p = 0,0066)**
PBI	6 – 10 years	1,91 ± 0,47	**2,62 ± 0,74 (p = 0,0469)**
	11 – 14 years	1,23 ± 0,59	**2,69 ± 0,75 (p = 0,0037)**
	15 – 18 years	1,49 ± 0,81	**2,72 ± 0,25 (p = 0,3320)**
CAL	6 – 10 years	0	**0,75 ± 1,5 (p = 0,167)**
	11 – 14 years	0	**1,6 ± 0,96 (p = 0,0013)**
	15 – 18 years	**1,033 ± 1,6**	**2 ± 1,8 (p = 0,399)**

In both, DS and control patients, the majority of the examined sites harbored dental plaque (Table II.1.2.2.1). Gingival bleeding was present at significantly higher degree in the DS young population, with a marked difference between the two groups, within the puberal age group (2,69± 0,75 in DS children, compared to 1,23± 0,59 in control group of the same age). Considering the third clinical indicator, no attachment loss was recorded in the prepuberal and puberal age in control group, while clearly higher levels were correlated to the intensive periodontal destructive process of DS patients, especially among juvenile subjects.

Thus, various degrees of periodontal attachment level were registered between the studied groups in the 15-18 years subjects (2 ± 1,8 in DS patients and 1,033 ± 1,6 among control; Figure II.1.29.).

- ***Assessment of the local level of some biochemical parameters in Down syndrome using gingival crevicular fluid as relevant inflammatory indicator***

Liver enzymes include aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and high concentrations in the blood tend to indicate liver disease. As evidenced by transient myeloproliferative disorder/abnormal myelopoiesi and neonatal cholestasis at birth, celiac disease-related and other autoimmunity-related liver diseases and gallstone formation due to gallbladder hypomotility, DS subjects are often affected by liver impairment (Park et al., 2014). Predisposition to major hepatotropic viral infections and/or a diminished response to the hepatitis B vaccine also have been reported, but the evidence is debatable.

As the liver is somehow frequently affected in persons with Down syndrome, our study upon DS individuals included either comparative measurements of the mean values

of gingival fluid AST (local levels) based upon dental pattern. The data with AST values among our studied groups is presented in Figure II.1.2.9.

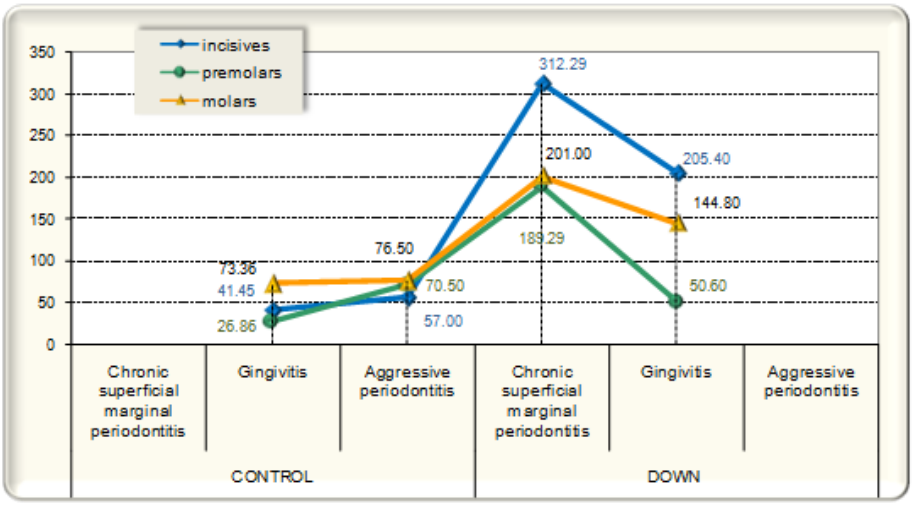

Figure II.1.2.9. *Variations of gingival fluid AST values in the studied groups, based on dental pattern and periodontal alteration.*

The Figure II.1.2.9 reveals the most elevated mean incisive AST value in the DS group compared to control, followed by molar and premolar levels, respectively. Thus, the mean AST levels differ significantly among DS children and adolescents, upon the dental pattern, the most significant variations being recorded between molars and incisives, and between incisives and premolars ($p << 0,05$), respectively.

Some pro-inflammatory cytokines are considered to play key roles in inflammatory responses. It has been reported that macrophages and T cells secret IL-6 to stimulate the inflammatory responses, while IL-1β promotes B cell maturation and induces immunoglobulin production, which eventually leads to inflammation (**Dinarello, 2000**).

Therefore, in our study we seeked for measurement of the level of the IL-1β inflammatory cytokine in subjects with DS, in hope of better understanding of the etiology of the diseases and potentially use this cytokine as biomarker for disease progression. The average levels for interleukin 1β from the GCF are presented in the Table II.1.2.2. The patients were grouped on the basis of periodontal and systemic health.

Table II.1.2.2. *The average level of gingival fluid IL-1β depending on the periodontal diagnosis in the studied groups'*

Group/IL-1β	Diagnosis	Case no.	Average IL-1β[ng/ml]	Standard deviation	Min	Max
CONTROL	Gingivitis	22	109.364	41.880	0.000	140.000
	Aggressive periodontitis	2	211.167	12.024	190.000	230.000
DOWN SYNDROME	Gingivitis	10	1272.133	659.601	0.000	1672.000
	Chronic marginal periodontitis	14	1824.905	420.479	0.000	1992.000

From the analysis of the data presented above, it results that systemically healthy patients with the mildest form of periodontal tissue alteration (respective gingivitis) recorded the lowest level of crevicular IL-1β, 109.364 ng/ml respectively (Table II.1.2.2).

The IL-1β values increased significantly in this group, concomitantly with the severity of the disease, resulting in an increament of approximately two folds (1.93) in the patients with aggressive periodontitis (211.16 *vs.* 109.36 ng/ml).

Considering the group of DS subjects, the results are spectacular, recording an increase of 13.53 times (p=0.000008) between the average value of IL-1β in the subjects with DS (1594.583 ng/ml) compared to control subjects (117.847 ng/ml).

II.1.2.4. DISCUSSION

Down Syndrome either known as trisomy 21 which better describes the anomaly was the first genetic disorder described in 1866 by John Langdon Down, representing still the most common genetic disorder (1/700 newborns).

DS individuals present anatomical abnormalities, mental and orofacial problems that potentialy impact the life-quality of life, younger patients seeming to present more positive attitude regarding supervised tooth brushing program compared to older patients. Additionally, older patients demonstrated mediocre practical skills. These two factors emphasize the importance of early preventive approaches in DS patients.

Subjects with disabilities can learn and perform tooth brushing procedures by themselves once are encouraged and motivated. According to some authors that have extensively focused upon the oral manifestations in DS individuals, most of them improved their motor capability and dexterity in brushing their tooth and developed self-care skills (Shyama et al., 2003). Use of alternative materials and methods (psychological support and social reinforcements) by dental hygienists and teachers seemed to demonstrate a positive and strong effect on these individuals, empowering their attitude concerning for patients with disabilities.

DS patients present mild to moderately reduced T and B cell counts, absence of normal lymphocyte expansion in infancy, suboptimal antibody responses to immunizations, decreased immunoglobulin A in saliva and neutrophil chemotaxis. Preventive approaches and treatment modalities of gingivitis and periodontitis include removal of dental biofilm, surgical and nonsurgical therapy. Preventive actions involve supervised brushing or stimulation of oral hygiene habits. Periodontal treatment, basically include scaling and root planing (surgical or non-surgical), associated or not with local and/or systemic antibiotics. Furthermore, participation of parents, caregivers and possibly institutional attendants are fundamental for the maintenance of the accomplished outcomes (Zandbergen et al., 2013).

Preventive methods and conventional periodontal treatments do not always result in the expected outcomes in this group of patients, requiring specific approaches. These factors justify the need of further research efforts, including more effective preventive and therapeutic procedures, with possible association with adjunctive chemical substances. Although several efforts are made to improve oral hygiene pattern in patients with disabilities, frequently mechanical actions solely are insufficient (Nizri et al., 2013).

Affected individuals often develop a form of aggressive periodontal disease that affects both temporary and permanent teeth (Shukla et al., 2014) and may lead to early expulsion of teeth, loss of alveolar bone measured on orthopantomography being found in 69% of patients with trisomy 21, according to some authors (Shyama et al., 2003).

Our results are in agreement with the ones above, the evaluation of clinical indexes of periodontal status (PBI, CAL) highlighting their increases in the group of children with Down syndrome.

Our main goal was a deeper understanding the pathogenesis of periodontitis in DS individuals as this would greatly help with the management and control of the destructive process associated with the disease and help DS affected individuals retain their teeth hopefully throughout their lifetime. Previously researchers have investigated factors usually associated with periodontitis such as subgingival plaque microbial composition, immune and inflammatory responses individually in DS affected individuals. The individual factors investigated were never collectively evaluated together to provide an overall understanding of the pathogenesis of periodontitis in DS subjects.

Periodontal changes are characterized by the formation of deep periodontal pockets, associated with increased bacterial plaque and intense gingival inflammation, in accordance with the results obtained, the maximum values recording levels as high as: QHI= 5, PBI = 4, CAL = 4 mm.

Oral hygiene is often used as a predictor of patients' caries experiences. Poor oral hygiene directly correlates to the degree of mental retardation as do increased rates of caries among those populations. Limited access to care, narrow manual dexterity, and reduced effectiveness of self home care are all elements triggering increased prevalence of gingivitis in those who have mental disabilities compared to controls. A possible conclusion is that the same should be true for the patient with DS; however, dental caries are less prevalent, researches upon aggressive periodontitis also revealing the absence of tooth decay despite high plaque levels, suggesting a correlation between DS mental disorder and severe forms of periodontitis (López-Pérez et al., 2002).

The high incidence of the disorder requires an understanding of physiologic, immunologic, anatomic, and microbiologic differences within this specific population in order to formulate the best treatment plan. DS patients with have various limitations that require consideration during diagnosis and therapy.

Studies examining the polymorphonuclear leukocytes' activity toward *Aggregatibacter actinomycetemcomitans* in patients with DS compared to age-matched controls clamed a significant decline in activity (Zhou & Windsor, 2006). The advanced tissue destruction seen in the DS population can, in part, be attributed to both endogenous and exogenous collagenase activity inductions, therefore, in our subjects, determination of metalloproteinases would be of great interest, besides evaluation of the transaminase. Matrix metalloproteinase-2, has been shown to be activated by *A. actinomycetemcomitans*, while levels of prostaglandin E2, leukotriene B4 and matrix metalloproteinase-9 in gingival crevicular fluid from DS individuals were higher compared to matched controls (Tiranathanagul et al., 2004).

Most authors reported the very early installation of periodontal degradation as well as a rapid and severe rate of destruction of the tooth support tissues (Cichon et al., 1998).

The results of our study are consistent with literature's data, emphasizing the critical periodontal damage in children with DS. The periodontal destructions are characterized by the formation of deep periodontal pockets, associated to increased quantities of bacterial plaque and intense gingival inflammation (maximum values QHI=5, PBI=4, CAL=4 mm), values which cannot be explained only on local factors (bacterial plaque, calculus).

Regardless of periodontal alteration (gingivitis or chronic marginal periodontitis), the gingival fluid levels of AST are higher in DS subjects compared to control group. Furthermore, levels of AST in GCF of DS group are almost 5 times more elevated than those from control subjects with mild periodontal status alteration - gingivitis (205,4U/l *vs.* 41,45U/l) (Figure II.1.2.9). Moreover, among DS children experiencing chronic marginal periodontitis, the gingival fluid AST recorded of about 7,53 more elevated values compared to control patients with gingivitis, and of 5,47 times higher than AST levels of control patients with aggressive periodontitis.

Based upon degree of periodontal impairment, comparative analysis of intracellular enzyme marker within GCF revealed significant differences in and between the studied groups (Figure II.1.2.9). Although not specific for liver disease, aspartate transaminase can be used in combination with other enzymes to monitor the course of various liver disorders; additionally, AST levels may be a useful adjunct in the clinical assessment of periodontal disease sites since AST gingival crevicular fluid level declines when periodontal status improves.

In our research, there is an important elevation of mean AST with severity of periodontal destruction, both in control and DS group, with significant elevation of enzyme level in aggressive periodontitis compared to gingivitis within the control group (76,5 *vs.* 73,36) and at higher levels within DS chronic marginal periodontitis compared to gingivitis (312,29 *vs.* 205,4).

Considering the immuno-inflammator status at the periodontal level, the comparative study on periodontal diseases underlined the the increase of IL-1β with the severity of periodontal disease (1824.925–chronic marginal periodontitis *vs.* 1272.133 – gingivitis), the level of significance recording the value p=0.000028. Moreover, comparing the averages values of the cytokine in DS patients with gingivitis individuals (1272.133 ng/ml) to those from the control group with gingivitis (109.364 ng/ml), a difference of 11.63 could be recorded, the level of significance of the comparing test according to a confidence interval of 95% being p=0.000026 (Table II.1.2.2).

The differences were even more noticeable when compared IL-1β from the subjects with Down syndrome - those with chronic marginal periodontitis (1824.905 ng/ml) to those with gingivitis from the control group (109.364 ng/ml) (because at these patients, chronic marginal periodontitis was not registered), recording an increase of 16.68 (p=0.000010). An important difference was observed between the value of IL-1β in chronic marginal periodontitis of DS subjects (1824.905 ng/ml) and IL-1β in active

periodontitis of control subjects (211.167 ng/ml), the increase being of 8.64 times (p=0.000032).

Periodontal degradations are established in temporary denture and continue to the permanent one, with a rapid progression, sometimes toward aggressive forms of the disease.

Various factors may be involved in the increased susceptibility to gingival and periodontal diseases among individuals with DS. Some of the previously investigated aspects comprise mental retardation, subgingival plaque composition, immuno-inflammatory responses, and microbiological circumstances, DS subjects displaying critical levels of periodontopathic bacteria such as *Porphyromonas gingivalis* and *Tannerella forsythensis*. Hence, optimal oral hygiene is of particular significance in these individuals in order to restrict the disease onset.

Dow in 1951 reported that more than 90% from the children with Down syndrome, having ages between 8-12 years old, develop some forms of periodontal disease. Subsequent studies also reported the prevalence of periodontal disease in percentages of 90-100% in the patients with 21 trisomy (Johnson & Young, 1963). Moreover, some reports claim that the periodontal index in the children with Down Syndrome was 4.5 times higher than in the healthy patients (Orner et al., 1976).

Although children and young adults with DS tend to have fewer caries because of some associated conditions such as delayed eruption of primary and permanent teeth, congenitally missing teeth, and microdontia, some may have increased risk of periodontal disease due to cariogenic food choices and reduced food clearance from the mouth.

Our results in patient statistic data support those from the literature, the prevalence of periodontal disease displaying less generalized bacterial gingivitis (33.33%) and more chronic marginal periodontitis, that represented almost two third of the total periodontal breakdown (66.67%). Numerous other studies have shown an increased prevalence of periodontal disease in patients with Down syndrome compared to other deficiencies (Johnson & Young 1963; Sznajder etal., 1968). Moreover, statistic data revealed increased prevalence of periodontal disease in hospitalized children with Down Syndrome compared to the non-institutionalized subjects (National Down Syndrome Society, 2015).

CONCLUSIONS

In young subjects with DS, periodontal breakdown is more important than dental caries. The incidence of periodontal disease in subjects with DS is very high, targeting 90-100%, with an age-related increase pattern, about 90% affected children and 96% DS adults being affected by periodontal impairment.

The present study revealed that the overall oral hygiene status of the study population was poor, very probably due to reduced manual dexterity of the DS subjects, joint laxity, and lack of comprehension of oral hygiene needs due to mental difficulties.

Moreover, despite that data from literature shows that in the patients with Down Syndrome aggressive forms of periodontitis are encountered, in our study-group affected by this mental disability, the most representative periodontal damage reffered to chronic

marginal periodontitis (66.67%), followed by gingivitis (33.33%), the aggressive forms of periodontal disease being absent.

The main limitation in the present study is the fact that the study was conducted on hospitalized DS children, whereas the majority of DS children still reside at home with narrow educational or vocational plan from parents as they are often stigmatized by the society. Therefore, generalization must be made with care, as this study group may not reflect the DS population in general.

- ***Considerations on ethical and religious implication in Down syndrome***

My contributions to this research direction can be found in the following articles:

1. Zegan G, Anistoroaei D, Cernei ER, Sodor A, **Toma Vasilica**. Assessment of schoolchildrens' knowledge, attitudes and behaviors for oral health. *Romanian Journal of Medical and Dental Education*, 2019; 8(2):64-72.
2. Maftei GA, **Toma Vasilica,** Filioreanu AM, Ciurcanu O, Popa C, Foia L. A case of bilateral impaction of maxillary incisors in an intellectually challenged patient. *Romanian Journal of Medical and Dental Education*, 2018; 7(2): 81-86.
3. **Toma Vasilica**, Foia LG, Forna D, Toma CM, Cioloca D, Balan G, Balan A. Down Syndrome between genetic hazard and divine decision. *European Journal of Science and Theology*, 2014; 10(3): 49-59.
4. Balan A, Balan G, **Toma Vasilica**. Religion, subjectivity and oral health ethical dilemmas in pediatric dentistry. *European Journal of Science and Theology*, 2014; 10(1): 155-165.
5. Maxim A, Savin C, Bălan A, Păsăreanu M, **Toma Vasilica**, Maxim DC, Petcu A, Serban V. The impact of socio-cultural model on the child education for oral health. *Romanian Journal of Oral Rehabilitation*, 2011;3(1): 43-47.

Found in the literature under the name of Langdon Down syndrome, Trisomy 21 (an adequate term, if one considers the ethiopathogenesis) or Mongolism (an inadequate term), this „mental disease" was first noticed by Esquirol in 1838; later on (1846, 1866), Séguin consecrates a special chapter to the „furfuraceous" cretinism in his book: " Moral treatment, hygiene and education of idiots and other children retarded in their development" .

In 1866 Langdon Down insists on the stereotypical physiognomy and behaviour of these sufferings. He defines the disease, differentiating it from the other forms of the mental debility, stating that "when these children sit together, one by one, it is hard to believe that the compared subjects are not the children of the same parents". He issues a theory according to which the mental retard of these children is caused by the repartition of the characters specific to the Mongolian race, hence the ascribed name of „Mongolian idiot". The chromosomal theory was confirmed in 1959 by the discovery made by Lejeune & Turpin of an excedent chromosome, therefore 47 instead of 46 chromosomes in these subjects (Lejeune & Turpin, 1959). This fact was also confirmed by Ford in 1959, on a case of trisomy 21 and recognized by all the cytogenetics laboratories and by the subjects

of all the races (Ford et al., 1959). In 1960, Johnson presents the first case of translocation (Johnson & Young 1963), and in 1961 Clarke reports cases of mosaic trisomy (Clarke et al., 1961).

- ***Clinical framework, physiopathology, etiopathogeny, epidemiology in DS***

The existence of the third chromosome 21 in DS affects most of the tissues and organs, through the appearance of numerous disease phenotypes. These include complications that can set the life in danger, significantly impairment of the life course (mental retard) and characteristic physical dysmorphia. Moreover, the Down syndrome was associated with multiple lethal complications that reduce the pre-natal viability and increase the post-natal morbidity and mortality. The affected children display retardation in their growth and maturation, mental retard, delays in bone development and teeth eruption.

Due to the immune adverse response, the DS-associated infections are frequent, as are the auto-immune diseases, such as Hashimoto thyroiditis. Moreover, due to the metabolic homeostasis alteration, the DS-affected children are predisposed to hyperuricemia and hyperglycemia, increased resistance to insulin, whereas the malfunctions of the hematogenous marrow favor the appearance of leukemia-type modifications (especially transitory myeloproliferative disorder and acute megakariocytic leukemia). In most children who developed leukemia, a mutation was noticed in the gene that encodes the hematopoietic transcription factor GATA 1. Development of these blood disorders in DS children is conditioned by three factors: trisomy 21, mutation at the level of gene GATA 1 and an yet undefined genetic alteration.

The recent studies back-up the hypothesis according to which the Trisomy 21 results from the super-expression of the genes localized on the chromosome 21, one of these genes being the one for superoxide dismutase (SOD), whose activity increases in DS; the SOD enzyme converts the superoxide anions (free radicals) in hydrogen peroxide and water. The radical species produced in excess at the cell level determine both functional and structural alterations; that is why an enzyme with the role of free radicals neutralization (such as SOD in this case) has an overwhelming importance.

The alteration of the genes located in the so-called „critical" zone 21 q22.1- 22.3 (also named DSCR - Down Syndrome Critical Region) is responsible for the generation of most of the clinical signs characteristic for the Down syndrome. Therefore, Trisomy 21 results from the meiotic non-disjunction at one of the parents. This is correlated with the mother"s and eventually father"s old age. The translocation can appear *de novo* or can be transmitted by one of the parents, involving the chromosome 14 (translocation 14/21), chromosome 21 (translocation 21/21) or chromosome 22 (translocation 22/21).

Anyway, with all the accomplished progresses, the DS pathogenesis still has many enigmas. Having the incidence of 1/700 births and currently affecting over 300000 individuals only in the United States of America, the trisomy 21 remains the most frequent chromosomal anomaly (Perluigi et al., 2014). The frequency of the products of conceptions with trisomy 21 is much higher (1:200), yet much of them do not have viability being removed through spontaneous abortion. The disease is more frequent in male children, the ratio being 3 boys to 2 girls.

The fact that the risk of giving birth to a child with Trisomy 21 increases with mother‟s age was recognized long ago, and the reports show that the risk to give birth to a DS child at 30 year is of 1 to 1000, while it increases to 9:1000, at the age of 40 years. That is why the mother‟s age of 35 was chosen as an indication of echographic and biochemical screening, as well as for the prenatal diagnosis (the risk of a foetus with DS being higher than the risk of abortion associated with the procedures of diagnosis, as well as the biopsy of chorial villosities or amniocentesis). A delicate problem is the risk in those families where there is a blood relation with DS or unknown caryotype.

Taking into account the trisomy frequency in terms of mother‟s age, as well as the possibility of a translocation, the highest calculated risk is of 1:640 (therefore close to the prevalence among the population).

- **Down syndrome and the laws of life**

The exact causes of the DS appearance are not known yet, which makes it different from the other genetic alterations, such as cystic fibrosis or falciform anaemia, which can be hereditary. The DS is hereditary only in a proportion of 1%, and these persons present Robertsonian translocation that implies the chromosome 21. Therefore, the essential problem in DS remains the elucidation of the aetiology of this frequent trisomy. The mother‟s age is the only evident determinant factor of the non-disjunction (Sujoy et al., 2009), yet only about 25% of the patients with DS are born from women over 35. Nevertheless, the fact that 80-90% of the cases result from maternal non-disjunction suggests the intervention of a risk factor that acts at this level (Yoon et al., 1996). The scientists claim that what is sure is that nobody is to blame, since up to now no environment factor that might contribute to the development of the affection was identified: “the appearance of DS is not caused by external factors, so that nothing of what a pregnant woman does during her pregnancy determines the disease”. The scientists do not know why sometimes the cells abnormally divide and produce the additional genetic material, which results after, in the DS occurrence.

Taking into consideration the heredity laws, we can state that life has its own mathematics, therefore its own explanation and thus one can intervene in its variables; moreover, considering the chromosome theory in biology, to what extent man can intercede in life organization, remains to be elucidated. If there is a possible explanation, it should be looked for in the cell structure, as any living being consists of cells and comes from a unique cell within which one must arrange all the hereditary factors that determine the individual‟s character; the possibilities to group the tens of thousands of genes are almost infinite, much beyond the human imagination.

Although many organs are affected by the DS phenotype, the central nervous system is a focus of study, partly because mental retardation is a key feature of the syndrome and partly because we know so little about the genetic causes of mental retardation even though ~1–2% of the population are affected in different ways. Classical anatomical studies, combined with new neuroimaging techniques of live individuals, are characterising the nervous system in DS, including the small cerebral and cerebellar hemispheres and brain stem. Histopathological studies show that the main difference

between DS and normal brains appears to be in neuronal organisation and number (Gardiner, 2014), including subtle alterations in different cortical layers, particularly reductions in cell number in layers 2 and 4.

Also in DS, the dendritic trees, which continuously expand in normal early growth and development, appear to become relatively atrophic 4 months after birth. Abnormal pre- and postnatal synaptic parameters have been reported, including possibly fewer synapses in DS and other changes that could lead to reduced efficiency of synaptic transmission (Hernandez & Fisher, 1996). A general delay in myelination and altered electrophysiological membrane properties have also been described for DS neurons.

And, if the Medicine - and generally the Science - could not establish yet who determines the DS, we wonder "What circumstance generates the gene recession such that to result in recessive genes? Where do these accidents of life come from?" Who keeps the book for the little infinity? Who performs the calculus of probabilities and has not finished the probabilities? What are the laws to which the maternal germinal cell is subjected to when, in the fecundation process, it removes through the two germinal cells, half of its nucleus, which means the reduction of the chromosomal formula from 2N to N or from 48 to 23-24 chromosome pairs? Does the same thing happen at the same time with the paternal cell which „breaks it neck" and suffers from the same chromosomal reduction?

The current chromosome 21 transcription map provides us with some interesting sequences with which to investigate the consequences of overexpression. Such studies invariably take place with transgenic mice so that the effects on the whole body can be evaluated. Mice are not human and, therefore, we can only use them to model dosage effects, rather than DS. Nevertheless, mouse models allow us to dissect biological pathways and mechanisms even though the outcome of overexpression might be different from what occurs in humans. In the future, other model systems, such as fly or yeast, are likely to become important to functional studies of human aneupoloidy, especially as many partial trisomies are known in these organisms.

II.1.2.5. CONCLUSIONS

Down syndrome (DS) represents the most frequent chromosomal disorder, determined by the presence of supernumerary chromosome 21. The existence of the third chromosome 21 affects most of the tissues and organs by the appearance of a suggestive craniofacial dysmorphia, various visceral malformations and psychomotor retard, the most common error being the maternal non-disjunction during the first meiotic division.

Despite all the progresses accomplished in genetics, the DS pathology has not been completely decoded, the advanced maternal age (over 35 yo) being at present the only determinant factor clearly responsible for the maternal meiotic non-disjunction.

Science can"t decide who gives the verdict in the fecundation process, reducing the chromosomal formula from 48 to 23 or 24 pairs of chromosomes: the hazard, the chance, or the laws- whose laws? Did the life give its own laws and is subjected to them?

Approaches in the human population can be taken to establish why the constant and variable features of DS occur—is trisomy for a region enough to have an effect? or are

particular allelic combinations important? and do epistatic effects (such as modifier loci) and environmental effects come into play?

All molecular genetic investigations depend on the genomic resources that are available and, fortunately for those researching the molecular genetics of DS, chromosome 21 is a paradigm for mapping and cloning studies.

II.1.3. Periodontal implications of immunodeficient status during neoplastic disorders in young children

II.1.3.1. STATE OF THE ART

Worldwide, in children and adolescents, cancer tends to exceed infectious diseases as a mortality cause. For children populations in Europe, North America and other developed regions of the world, incidence rate of cancer is about 140 in 1 million. In the United States, cancer is the second mortality cause in children, after accidents (Jemal et al., 2007). According to the European Society for Pediatric Oncology (SIOPE), 15.000 new cases are diagnosed every year in Europe, and each year over 3.000 children and adolescents die of cancer, which still remains the leading annual cause of death by disease in Europe (Steliarova-Foucher et al., 2004). In Romania, an incidence of 7,35 in 100.000 children and a distribution of types of tumors similar with international statistics has been reported (Cancer situation report in children in Romania, 2015).

According to the literature data, almost 30% of children cancers are leukemias (Smith, & Ries, 2002). Moreover, in Brazil, leukemia is the main cause of death by cancer in children (Pinheiro et al., 2016).

The classification of leukemias is complex, and all the types can be found in detail in the World Health Organization's classification of hematopoietic and lymphoid tissues tumors, published in 2001 and revised in 2008 (Campo et al., 2011). Leukemias can be thus generally classified based on the primary cell line of origin in: lymphoid or myeloid leukemia. Further, according to their evolution, leukemias can be divided into: acute or chronic (Burke et al., 2008). hence, one can distinguish 4 main types of leukemia: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphoid leukemia (CLL) (McCord et al., 2017).

Acute lymphoblastic leukemia is the most common form in children, consisting in about 75% of the newly diagnosed leukemias and 25% of all malignant diseases of the child (Campo et al., 2011). The etiology is still uncertain, but a few causal factors have been proposed: viral infections, exposure to ionizing radiation and chemicals (benzoic derivatives, heavy metals, pesticides) and also immunologic factors (Carroll et al., 2003). Some studies indicate environmental factors like: smoking and alcohol consumption in parents, bacterial infections, chemical substances, exposure to electromagnetic radiation and electric fields, as well (Eden, 2010).

The purpose of this section was to scrutinize the existing literature for the current data regarding the correlation between oral manifestations and leukemia in children, with strength upon the most common gingival and periodontal signs observed in children with leukemia.

My contributions to this research direction can be found as follows:

1. **Toma Vasilica**, Adumitroaie A, Cioloca D, Golovcencu L, Foia L, Halitchi LG, Zegan G, Gingivo-periodontal manifestations in children with leukemia: a literature review. *The Medical-Surgical Journal*, 2019.
2. Adumitroaie A, Foia L, Anistoroei D, Cioloca D, Maftei G, Bogdan M, Vlad C, **Toma Vasilica**. Review of the correlation between specific biomarkers in leukemia and periodontal disease in children. *Romanian Journal of Medical and Dental Education*, 2019; 8(3): 23-27.

II.1.3.2. MATERIALS AND METHODS

We accessed Articles from Medline database (via PubMed) using MeSH search terms:

- ”oxidative stress markers” and ”leukemia” and ”children” and ”periodontal disease”;
- „oral manifestations” and „leukemia” and „children” and „gingival manifestations” and „periodontal manifestations”.

The literature search continued manually, for printed articles and publications with the same criteria as with the electronic search. Literature was evaluated also by searching printed studies, with the same examination criteria as for the electronical search.

II.1.3.3. RESULTS AND DISCUSSIONS

Historically, the connection between specific oral signs and acute leukemia was first noted in 1964, in a study which reported that 20% of acute leukemia patients also presented „buccal lesions”, observing that 58% of acute leukemia subjects enrolled in the study, presented oral signs of the disease (Lynch & Ship, 1967).

In a study of Tagaki, 50% of the 16 patients with acute leukemia studied were first seen by a dentist, for oral problems (Tagaki et al.,1978). Stafford reported that dentists were responsible for referring patients to hematological tests, which later led to diagnosing leukemia based on suspicions raised by oral manifestations in a quarter of the patients with acute myeloid leukemia and a third of patients with acute myelomonocytic leukemia (Stafford et al., 1980). All these papers evaluated the correlation between leukemia and oral manifestations, disregarding age.

In present time, modern oncology requires the presence of the dentist in each treatment phase and even before the diagnosis, for the management, maintenance of oral health and life quality of patients with leukemia (Mescua et al., 2017).

The oral cavity represents, to some extent, the interface between oral and systemic health, some oral diseases being able to affect general health, or reflect a manifestation of systemic homeostasis (Casamassimo et al., 2018). For instance, multiple caries may lead to difficulties in the growth and development of the child (directly by nutrition, or indirectly by chronic pain and its consequences). Vice versa, it is known that systemic diseases may alter oral health status by defectuous dento-facial development, altering the oral physiology and compromising patient's ability to maintain oral health (Casamassimo et al., 2009).

Schlosser noted that the mechanisms of oral pathology in hematological diseases like leukemia may be direct infiltration through abnormal hematological cells, formation and deposit of abnormal proteins, oral ulcerations and abnormal hematopoiesis (Schlosser et al., 2011).

According to Philipone, clinical manifestations of leukemia in general, result from loss of normal leukocyte function, suppression of hematopoietic cell lines, or by direct infiltration of leukemic cells in tissues (Philipone, 2017). Thus, systemic signs and symptoms may include: fatigue, anemia, lymphadenopathy, recurrent infections, bone and abdominal pain, bleeding and petechiae. Oral manifestations involve the mucosa and may be: gingival bleeding, petechiae, ulcerations. Patients are also susceptible to severe viral, bacterial or fungal infections, as a consequence of immunosuppression. Gingival leukemic infiltrate leads to edematous, erythematous and friable gingiva.

McCord described the main oral signs and symptoms in children with leukemia: neutropenic ulcerations, spontaneous gingival bleeding, opportunistic infections (like: *Candida albicans*), and in chronic lymphoid leukemia – the possibility of developing paraneoplastic pemphigus (McCord et al., 2017). Moreover, Hou have shown that oral manifestations may appear in any type of leukemia, but are more prevalent in acute leukemias (as in chronic leukemias) and myeloid leukemias (as in lymphoid leukemias) (Hou et al., 1997). In his study, McKenna suggests that deep, painful oral ulcerations are common in leukemic patients (McKenna, 2000). Some data in the literature stated that oral examination of patients with leukemia may reveal mucosal pallor due to anemia, or bleeding and petechiae in the palate, lingual or labial regions, as a result of thrombocytopenia (Eisen et al., 1998).

In a recent study, Casamassimo describes several oral manifestations in lymphoid leukemia, as following: muco-gingival pallor, purpura, spontaneous gingival bleeding, necrotic ulcerations, herpes infections, candidiasis, bacterial infections, dental mobility, aggressive periodontitis, periapical radiolucency, paresthesia, facial edema, lymphadenopathies in head-neck region. For the myeloid leukemia, the author reports diffuse gingival enlargements and masses of soft edematous tissue in the oropharyngeal region (Casamassimo et al., 2018).

In children and adolescents, clinical aspects and manifestations of periodontal diseases are different than those seen in adults, one of the major differences being gingivitis (it is more frequently seen in children, whereas periodontitis develops more rarely). Such differences disappear in children with associated systemic diseases, particularly leukemia.

In 2012, Pels have studied 156 children (78 with acute lymphoid leukemia and 78 with unaltered systemic homeostasis) from rural and urban regions in Poland (Pels et al., 2012). They have analyzed oral hygiene status and the results suggested a precarious oral hygiene in children with leukemia from rural regions, compared to leukemic children in urban regions. The oral hygiene was better in children with acute lymphoid leukemia compared to healthy subjects, because of their rigorous oral hygiene regime. However, the gingival indices evaluated (simplified oral hygiene index – OHI s, plaque index – PI and gingival index – GI) were more elevated in leukemic children, indicating a higher risk of mucositis, despite a more important preoccupation for maintaining oral hygiene. In a previous study from 2007, the same authors have reported similar results – they noticed a better oral hygiene and a lower incidence of plaque in children with acute lymphoid leukemia.

Hedge have evaluated periodontal health status in 120 children with and without acute lymphoid leukemia (Hedge et al., 2011). The authors have noticed the presence of gingival inflammation more often in children with ALL than in healthy subjects. Ponce-Torres et al. have conducted a study in 2010 on 49 children with ALL, reporting a prevalence of 91,84% for gingivitis and 16,32% for periodontitis in these children. Literature data are somewhat diverse, especially considering the geographical distribution of populations, some reports from the beginning of this millennium indicating a much lower incidence, of 10-17% for these gingival manifestations in children with leukemia (Curtis, 2001; Michaud et al., 1997).

Some authors have reported, as the most common oral manifestations in children with leukemia, the following: gingival bleeding, hyperplasia, opportunistic infections and bone impairment (Morais et al., 2014). Previously, the most common oral manifestations observed were gingival edema caused by leukemic infiltrate, gingival hyperplasia usually being generalized and different in severity (Kaste et al., 1997).

In a study including patients with acute lymphoid leukemia, the most suggestive signs of leukemia revealed were: lymphadenopathy in the head-neck region, along with gingival bleeding and mucosal pallor (Baliga et al.,1995). In another study of 77 patients with ALL, poor oral hygiene, infections due to medullary and immune system suppression and altered healing responses were reported (Michaud et al., 1977).

In a recent study on 3789 children under 12 years of age, the authors reported that oral manifestations were a big contributor to the diagnosis of leukemia for 6% of the patients (Nakhostin & Meighani, 2016). The most common oral signs observed were: gingival bleeding, petechiae, ecchymosis, mucosal alterations and fungal lesions. In another study, gingival bleeding was observed as a first symptom of leukemia in children for 17% of acute cases and 4% of chronic forms (Vural et al., 2004).

The importance of a complex systemic evaluation in the context of suggestive oral signs is also underlined in a case study (Safari et al., 2016). Along other unspecific general symptoms such as bone pain, a 9 yo patient presented to the dentist for dental pain, dental mobility, erythema and gingival edema. He was later referred to the pediatric and hematology department, where the suspicion for lymphoid acute leukemia was confirmed. In 2002, Katz & Peretz have reported a case of acute lymphoid leukemia diagnosis based

on the presence of dental trismus (Katz & Peretz, 2002). The trismus was probably caused by the infiltration of leukemic cells in the deep portions of facial muscles. With their study, the authors underlined the importance of clinical and laboratory exams recommended by the dentist.

Distinctive signs of acute necrotizing ulcerative gingivitis, associated to acute lymphoid leukemia have been identified: patient presented the classic triad of pain-bleeding-ulceration, along with secondary signs like subfebrile state and decapitated gingival papilla, covered with pseudomembranes (Kolli et al., 2014).

In present, there are tendencies in the literature to systematize, in a standardized fashion, the most common oral manifestations in general, and gingivo-periodontal manifestations in particular, observed in children with leukemia.

The dentist should know and differentiate these signs and symptoms during the protocols for clinical examination. This would allow a higher success rate of an interdisciplinary early diagnosis issued by the cooperation between the dentist and pediatrician/hematologist, for children with leukemia. To accomplish these needs of standardization and systematization, further studies are required.

Numerous circulating biomarkers can indicate a pathological state in leukemia. Malignancy is associated with an elevated oxidative stress and decreased antioxidant factors. Various biomarkers, such as hematological, hepatic or renal indicators, as well as oxidative stress factors, electrolytes and vitamins (C, E) can be investigated. In their study, some researchers revealed the presence of an elevated oxidative stress level, decreased levels of enzymatic and non-enzymatic antioxidants in adult leukemia patients, reflecting a pathological condition and an altered cellular control (Rasol et al., 2015).

Oxidative stress is one of the potential malignancy mechanisms, due to the mutations that free radicals seem to produce in the DNA, resulting in neoplastic alterations. On the other hand, paradoxically, a high antioxidant defensive activity may stop the elimination of mutant cells and increase the development of a neoplastic modification.

Immune salivary factors play a very important role in maintaining normal functions of the oral mucosa. Patients with leukemia in general, and those under chemotherapy in particular, often have salivary alterations and a tendency to develop inflammatory conditions of the oral mucosa. Alteration of the immunological homeostasis leads to the development of pathological lesions described as mucositis. Oral mucositis is a complex pathology, resulted from the interaction of antineoplastic agents with the epithelial cells, actions of the proinflammatory cytokines, oral microbiota, overlaying local trauma, unsatisfactory oral hygiene and poor immunological status (Pinto et al., 2006). Initially, mucositis is observed as an erythematous plaque, developing into an ulceration. Oral mucositis is associated with pain, which leads to eating difficulties and speech impairments, that can go to cachexia. Moreover, these lesions can be a gateway to opportunistic infections: fungal, bacterial or viral (Valera et al., 2015).

In order to evaluate biomarkers in various pathologies, including some serious conditions, saliva analysis is increasingly more used. Some comparative studies suggest that saliva analysis can be an alternative to blood analysis in the evaluation of specific

biomarkers (Pels, 2015). Due to the non-invasive collecting method, saliva analysis may be easily used in children, or in patients that suffer not only from the primary condition, but also from leukemic complications (Khalaf et al., 2014).

Other researchers had revealed the presence of significantly high levels of IL-1β, IL-6, IL-10 in children with acute lymphoblastic leukemia. Moreover, the authors reported decreased levels of IL- 2, TNF-α and IL-4. Saliva is the first defense line against free radicals mediated by oxidative stress (Du et al., 2014). Hedge have discovered a decrease in salivary flow, salivary pH and global levels of salivary antioxidants, in children with leukemia comparative with healthy children (Hedge et al., 2011). Their results suggest an alteration of oral status and gingival tissues, as well as an increased carious activity in children with leukemia, which implies the necessity of an interdisciplinary approach for the treatment of these patients.

More recently, the role of the immunological system in the homeostasis of oral status and structuring the oral microbiota, highlighted by salivary tests in healthy and leukemia children have been issued (Wang et al., 2014). They have detected a structural imbalance, characterized by a reduced oral microbiota and an increased level of altered bacteria, which are involved in systemic infections.

Since periodontal disease is a very common oral pathology in patients with leukemia (Javed et al., 2012), it is crucial to distinguish whether the modification of biochemical markers is caused by the alteration of immunological system, or the preexistent periodontal pathology.

Numerous studies suggest that periodontal disease contributes to the local oxidative stress, and also to the systemic oxidative stress. Lipid peroxidation, protein and DNA alteration can be used as biological markers of oxidative stress associated with periodontal disease. Local and systemic activity of some antioxidant factors can, also, be influenced by periodontal disease.

In normal physiological conditions, there is a balance between oxidative activity and antioxidants. Oxidative stress appears only when antioxidant defensive systems cannot neutralize the increased production of oxidative factors (Sies, 1997).

Antioxidants are grouped in 2 main categories, by their type of action (Chapple et al., 2007): preventive antioxidant activity, like enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase and DNA repair enzymes, such as some metal ions sequestrants like albumin; the second antioxidant category is represented by cleaving antioxidants, like ascorbic acid, carotenoids (including retinol – vitamin A), uric acid, α-tocopherol (vitamin E), reduced glutathione and polyphenols (flavonoids).

The activity of superoxide dismutase (SOD) and catalase (CAT) have been investigated in gingival tissues and were found to be declined in accordance to the depth of the periodontal pocket (Ellis et al., 1998).

Periodontal disease is accompanied by high levels of lipid peroxidation and alteration of antioxidant status, alongside the depletion of antioxidant action of uric acid, reduced glutathione, vitamin C and α-tocopherol. Oxidative stress in periodontal disease,

even if it is not an etiological factor, contributes to its affliction. Oxidative stress markers record high values both, in saliva and blood (Nănescu et al., 2006).

MDA (malondialdehyde) is the most studied marker that indicates an increase of the oxidative stress status (Monisha et al., 2016). Some studies have shown critical elevation of superoxide dismutase activity in periodontitis, which suggests a proportional increase with the intensity and progression of inflammation (Akalin et al., 2005). Moreover, it was suggested that it is improbable that oxidative process has a causal role in the etiology of periodontal disease, but it is likely to contribute to the disease progression (Nanescu et al., 2006). Crevicular fluid would add even more oxygen reactive species, determining a blind loop, which worsens the situation of the gingival status.

II.1.3.4. CONCLUSIONS

Considering the growing prevalence of these diseases, children leukemia must be approached in a complex generalized systemic manner, in which the dental practitioner has a significant contribution. He often is the first one to observe signs and specific symptoms of the disease in the dental office. Among these oral signs, the gingivo-periodontal manifestations are the most common, therefore every dentist must know and be able to connect their presence in the oral cavity with this severe condition.

In periodontal disease, it has been shown that superoxide dismutase and catalase activity are criticaly diminished, accompanied by an increase of oxidative stress, and a decline in antioxidant capacity.

Also, it has been suggested that, although oxidative stress has no causal role in the etiology of periodontal disease, it probably contributes to the progression of the disease and the worsening of the gingival status.

Taking into consideration that narrow studies confirm the association between biochemical markers (salivary, hematological, hepatic or renal factors, as well as oxidative stress factors, electrolytes and vitamins) and periodontal disease and leukemia, but with scanty evidences which follow this correlation in children with leukemia and periodontal damage, further studies are required in order to document the interdependency of these factors and their clinical relevance.

II.2. GINGIVAL FLUID – SMOOTH INTERFACE IN THE EVALUATION OF THE PERIODONTAL STATUS

II.2.1. STATE OF THE ART

The gingival crevicular fluid (GCF) is a liquid substance found within the gingival sulcus, which originates from the blood circulating inside the vessels of the gingival connective tissue (corium). Due to the difference in osmotic pressure between the blood vessels and the rest of the gingival corium, the gingival fluid is secreted inside the gingival sulcus, through the internal and junctional gingival epithelium (Khurshid, 2017). In a healthy periodontium, the GCF should be found in a reduced quantity, having similar composition as other serous exudates. Physiologically, the GCF is mainly composed of plasma, aminoacids, carbohydrates, proteins, cellular elements and minerals (calcium, potassium, sodium) (Delima, 2003). The proteins found in the GCF are varied and include albumin, immunoglobulin, fibrinogen and different enzymes, such as phosphatase, hyaluronidase, protease, matrix-metalloproteinase and lysozyme (Uitto, 2003). The cellular elements of the GCF are represented by cells originating from the gingival epithelium, as well as defensive cells as lymphocytes, plasma cells and peripheral blood mononuclear cell. Bacterial cells of the subgingival bacterial plaque can also be found in the GCF (Delima, 2003).

The main role of the GCF is to combat subgingival bacterial biofilm accumulation, by mechanical, biological and chemical means (Griffiths, 2003). Mechanically, the GCF can simply wash away food particles or small bacterial deposits that may accumulate inside the gingival sulcus. For this reason the quantity of secreted GCF gently increases during or after mastication. Biologically, the GCF is rich in immune cellular elements and antibodies that can target and eliminate bacterial aggressors. The GCF also contains important enzymatic equipment, mainly lysozyme, which is able to weaken and damage the bacterial cellular wall and thus to chemically eliminate the bacterial challenge to the periodontal tissues (Surna, 2009).

The flow of the GCF has been observed to increase in various situations, as during the morning or when the gingiva is mechanically stimulated as by mastication, massage or brushing (Goodson, 2003). It may seem that certain hormonal changes can also impact the rate of GCF secretion, as its quantity slightly increases in pregnant women or when using oral contraceptive medication, as a result of elevated vascular permeability that these hormones induce (Becerik, 2010). Conversely, the peripheral vascular flow can be significantly reduced in smoking patients, also causing a reduction of GCF secretion levels (Morozumi, 2004). Nevertheless, in a healthy periodontium the GCF should be found in low quantities (Goodson, 2003). Despite its reduced flow, it seems that the GCF has to ability of enhance the adhesion of the junctional gingival epithelium's cells to the tooth surface, through its plasma protein content (Pollanen, 2003). Its presence inside the gingival sulcus can also influence the formation of subgingival calculus deposits, as it comes into contact with the subgingival bacterial biofilm (Teles, 2010).

The GCF was observed in patients as early as the 19th century, but thorough research on its forming mechanisms and role was only done in the middle of the 20th century, by Brill and Krasse, who injected a certain pigment (fluorescein) into the blood stream of dogs (Brill, 1958). After only three minutes the dye could be observed within the GCF of injected dogs, proving the plasmatic origin of the fluid. This led to the general belief that the GCF is a transudate, deriving from blood (Brill, 1962). However, as the

quantity of the secreted GCF increases exponentially during gingival and periodontal inflammation, recently this opinion has shifted towards the GCF being more of inflammatory exudates, rather than a transudate (Engelberg, 1966).

During periodontal inflammation, not only the quantity of secreted GCF increases, but its composition also changes. The quantity of secreted GCF increases because the blood vessels inside the inflamed gingival corium expand, allowing more plasmatic content and immune cells to reach the place of bacterial challenge (Giannopoulou, 2003). As it travels through the inflamed gingival tissues, the fluid becomes rich in leukocytes, bacterial cells or degraded epithelial cells, resulting in its purulent appearance, in the case of severe periodontal inflammation. Thus, it could be inferred that the compositional characteristics of the GCF are influenced by the level of periodontal inflammation. As a result, certain components of the GCF could be used a way to assess the inflammatory status of the periodontal tissues, in terms of severity, resolution or reoccurrence (Teles, 2010).

Various attempts have been at identifying an element that would reflect the inflammatory periodontal status within the GCF of periodontal patients. Such elements can originate either from the patient, as do immune cells or pro-inflammatory markers, or from the bacteria inflicting periodontal damage, as enzymes and bacterial toxins (Lamster, 1992). Nevertheless, this process is quite difficult, as the origin of some elements, particularly enzymes cannot be certainly tracked back to bacterial or host sources. For example, collagenase enzymes are secreted by host cells (neutrophils) and also by bacteria. In what concerns other compounds of the GCF, of organic nature, the results have not been conclusive (Sorsa, 1990). The GCF carbohydrate concentration (mainly glucose) is up to four times higher than that of blood, particularly due to the local specific conditions of bacterial plaque accumulation (Hara, 1969). Conversely, the protein concentration of the GCF is lower to that of blood, but research has offered no correlation between their levels and the periodontal inflammatory status parameters, such as pocket depth and bone loss (Curtis, 1988). More encouraging results have been obtained by assessing the correlation between the mineral components of GCF (sodium/potassium ratio) and the periodontal inflammatory status, suggesting some degree of significance (Koregol, 2011).

Prostaglandins (mainly prostaglandin E_2 – PGE_2) are a class of inflammation mediators that are secreted by monocytes in periodontal diseases patients and inflict alveolar bone loss, by promoting osteoclast activity. In GCF samples originating from patients suffering from periodontal disease, with important clinical attachment loss and alveolar bone resorption, the PGE_2 levels were higher than those of the control group, suggesting an active involvement in periodontal disease pathogenesis, which can be assessed by means of GCF analysis (Kumar, 2013). Matrix-metalloproteinases are zinc-dependent enzymes that are involved in the formation and degradation of collagen. Since collagen is one of the most important building blocks of the periodontal tissues, any disruption in the activity of the MMPs will result in impaired collagen formation and degradation processes, causing serious damages to the integrity of the periodontium. During periodontal inflammation MMPs activity is exacerbated and their inhibitors lack efficiency, resulting in a degradation of the collagen matrix of the periodontal structures by these enzymes (Sapna, 2014). Stimulated by bacterial endotoxins, such as the lipopolysaccharide, neutrophil cells will release increased quantities of MMPs, mainly MMP-8 and MMP-9 which aim type 1 collagen. This type of collagen is mainly found within the periodontal ligament. These important enzymes for the degradation of periodontal structures exhibit elevated levels in GCF samples of affected samples and they can correlate to the level of alveolar bone resorption and gingival clinical attachment loss,

further supporting the hypothesis that GCF can be used an assessment tool for periodontal disease status (Leppilahti, 2014).

The concept of "periodontal medicine" states that there exist numerous connections between the periodontal structures and pathology and the systemic conditions manifesting in one patient (Pizzo, 2010). These systemic conditions include diabetes mellitus, cardio-vascular disease, rheumatoid arthritis, renal and hepatic diseases and certain cognitive disorders. A part of the connections existing between periodontal and systemic conditions can have a bi-directional nature, suggesting the mutual influence that they can manifest on each other (Linden, 2013).

In periodontal patients with type 2 diabetes mellitus, the GCF levels of certain cytokines, including IL-1β, IL-6 and TNF-α were significantly higher than those of non-diabetic periodontal patients (Javed, 2012). Given the implications that these cytokines have in promoting the inflammatory reaction, it would be understandable to use GCF samples to assess the severity of the periodontal disease, in correlation with the clinical assessment of the disease's manifestations, which are known to be more significant in terms of severity and extent in diabetes patients. GCF sampling can also be useful for the assessment of the ratio between pro-inflammatory and anti-inflammatory cytokines in diabetic periodontal patients. In these patients, this ratio is often misbalanced in favor of the pro-inflammatory cytokines (TNF-α/IL-4, IL-1β/IL-4, IL-6/IL-4, TNF-α/IL-5 and IL-6/IL-5 ratios). As the secretion inflammatory mediators stimulates the activity of MMPs, in diabetic periodontal patients these enzymes become hyperactive, leading to rapid and extended periodontal tissue loss, as reflected by the elevated levels of MMPs in the GCF samples of such patients (Barros, 2016).

Cardio-vascular diseases, including coronary heart disease, ischaemic cerebrovascular disease and peripheral vascular disease are believed to possess an important inflammatory component as part of their pathogenesis. This is mainly true for the process of atherosclerosis, which blocks arterial vessels and leads to cardio-vascular events. More exactly, the presence of pro-inflammatory mediators can trigger the proliferation of smooth muscle cells and apoptosis of endothelial cells, leading to endothelial injuries, which are the start point of athermanous plaque formation. Thus, the pre-existence of an inflammatory background caused by chronic periodontal disease can create favorable premises for the development of cardio-vascular diseases (Schenkein, 2013). Moreover, it has been shown that the treatment of periodontal disease decreases the risk of cardio-vascular events, by reducing the load of pro-inflammatory mediators generated by the chronic inflammatory periodontal reaction (Pradeep, 2011). In this manner, the assessment of the periodontal inflammatory status by means of GCF pro-inflammatory mediators quantitative and qualitative analysis can be of real use for patients who exhibit additional risk factors for cardio-vascular diseases and pathologic events, such as smoking, high cholesterol, obesity and physical inactivity (Berry, 2012).

Rheumatoid arthritis, a chronic autoimmune degenerative disease causes inflammation around the joints, which become very painful and gradually immobile. This autoimmune inflammatory reaction is fuelled by pro-inflammatory mediators, including IL-1β, IL-17 and TNF-α. Obviously, the same pro-inflammatory mediators are also involved in the pathogenesis of periodontal disease. When the two diseases occur in the same patient, their inflammatory reaction can become synergetic, exacerbating each other (Detert, 2010). Moreover, the two diseases also include similar profiles of lymphocyte populations, as well as intensive activity of the MMPs, that trigger the degradation of periodontal structures and joint components, respectively. Therefore, the assessment of MMPs in GCF samples of affected patients, could be used a simple method of monitoring the disease's progression (Esen, 2012).

When dealing with infectious disease, such as viral hepatitis C, the GCF has been studied as a possible mean to transport viral antigens or antibodies. The hepatitis C virus (HCV) can multiply inside peripheral blood cells, which can be transferred from the blood stream into the GCF. In GCF samples originating from HCV infected patients both viral RNA and anti-HCV antibodies have been identified (Matičičr, 2001). During periodontal inflammation, the production rate of GCF increases, so the probability of HCV-infected cells to pass into the gingival sulcus consequently increases. For example, 59% GCF samples of HCV-infected patients tested positive for HCV RNA detection and 83% for anti-HCV antibodies (Suzuki, 2005; Açıkgöz, 2009). Moreover, the concentrations of HCV RNA were higher in GCF samples of infected patients than in saliva samples originating from the same patients, suggesting that the source of saliva contamination with HCV RNA in viral hepatitis C patients is, in fact, the GCF. However, this does not endorse the infectious potential of HCV RNA-loaded GCF, as this is highly influenced by other viral-load factors when an infectious contact occurs (Gheorghe, 2018). As viral hepatitis C implies the onset of an inflammatory reaction, GCF samples of infected samples exhibited elevated levels of pro-inflammatory mediators, including IL-1, IL-6 and interferon-gamma, similar to those of periodontal patients (Yu, 2012). The connection between chronic hepatitis C and periodontal disease, as suggested by the GCF analysis is also endorsed by the elevated levels of aspartate transaminase (AST) found in GCF samples of periodontal patients. This hepatic enzyme can be used as an indicator of periodontal disease evolution, showing improvement after periodontal disease treatment. It is also one of the first serological parameters that significantly increase during HCV infection (Shimada, 2000).

My contributions to this research direction can be found in the following articles:

9. **Toma Vasilica**, Cioloca DP, Forna DA, Hurjui L, Botnariu G, Nechifor IE, Bogdan M, Costuleanu M, Simion L, Holban C. IL-18 as an important gingival inflammatory biochemical marker in children and adolescents with insulin-dependent diabetes mellitus. *Rev Chim*. 2016; 67(12):2545-2551. (IF= 1,232)
10. Rauten AM, Silosi I, Stratul SI, Foia L, Carmen A, **Toma Vasilica**, Cioloca D, Surlin V, Surlin P, Bogdan M (equal contribution). Expression of Pentraxin 3 and Thrombospondin 1 in Gingival Crevicular Fluid during Wound Healing after Gingivectomy in Postorthodontic Patients. *Journal of Immunology Research*, 2016: 1-7, Article Nr: 4072543. (IF=3,276)
11. Gheorghe DN, Foia L, **Toma Vasilica**, Surdu A, Herascu E, Popescu DM, Surlin P, Vere CC, Rogoveanu I. Hepatitis C Infection and Periodontal Disease: Is there a Common Immunological Link? *Journal of Immunology Research*, 2018. Article Number: 8720101, DOI: 10.1155/2018/8720101. (IF 2017= 3,298)
12. **Toma Vasilica**, Goriuc A, Cioloca D, Nechifor I, Surdu A, Maftei G, Foia L, Filip F. The role of malondialdehyde in evaluation of the oxidative status of periodontal impairment. *Romanian Journal of Medical and Dental Education*, 2017; 6(1): 6-14.
13. Gheorghe DN, Camen A, Foia L, Solomon S, **Toma Vasilica,** Mateescu OG, Surdu A, Rogoveanu I, Surlin P. Histologic and immunohistochemical assessment of gingival tissue's changes induced by periodontal disease in association with chronic hepatitis C. *The Medical-Surgical Journal*, 2018; 112(4): 789-797.

14. Foia L, **Toma Vasilica**, Ungureanu D, Zlei M, Indrei A, Forna D, Filip F, Nanescu, S. Evaluation of the oral injuries in experimental induced diabetes mellitus by analysis of some gingival fluid markers. *The Medical-Surgical Journal*, 2008; 112(4):1066-1071.

II.2.2. MATERIALS AND METHODS

One of the direction of study referred to the investigation of interleukin 18 (IL-18) levels in the GCF, in young subjects with insulin dependent diabetes – IDDM, in an attempt to assess the possibility to use it as a marker of the immune-inflammatory disequilibrium in these subjects. We selected a number of 60 young subjects, aged between 7-18 years, 30 subjects with type I diabetes (IDDM, from Diabetes and Metabolic Diseases Department of the University Clinical Hospital St. Mary in Iasi) and assigned as active group, and 30 healthy subjects selected from children that addressed to the Clinic for Pediatric Dentistry, with various degrees of periodontal impairment. Exclusion criteria: previous orthodontic treatment, current cigarette smoking, periodontal and antibiotic therapies in the previous 6 months, systemic condition other than diabetes and subjects that presented diabetes complications (other than periodontal injury), that could possibly influence cytokine level.

○ *Periodontal assessment*

All the patients had periodontal evaluation using a periodontal probe 3.5/5.5/ 8.5/11.5 mm and Tweezer kit (Kerr-Total/Metrex research, Hamburg, Germany). Clinical investigation aimed to evaluate four periodontal variables:

- *Plaque Index* (PI) - Silness and Löe method,
- *Gingival Index* (GI, table II.2.1), as it reflects the qualitative changes in the gingiva, by scoring the marginal and interproximal tissues separately on the basis of 0 (normal gingiva) to 3 (severe inflammation).

Table II.2.1. *Gingival index*

GI = 0	Health Normal gingiva
GI= 1	Minor inflammation- narrow colour change, negligible edema No bleending on probing
GI= 2	Moderate inflammation- redness, shining, edema Bleeding on probing
GI= 3	Severe inflammation- redness, edema, ulceration Move toward spontaneous bleeding

- *Bleeding on probing* (BOP) was determined through gentle probing of the hole of the gingival crevice on 4 surfaces (mesial, distal, vestibular, lingual) of each tooth.
- *Probing Depth* (PD) was measured following insertion of the probe along the teeth axis on mesial, distal, buccal and lingual sites. For all subjects, the percentage of sites with values over 3 mm have been estimated;

- *Clinical attachment level* (CAL) represents the length between the cemento-enamel junction and the bottom of the groove, the percentage of sites with value > 2 mm, indicating a loss of bone support being evaluated for all enrolled subjects.

Based on their periodontal status, each subject of the active and control group was included in the gingival healthy group or gingivitis group according to the following criteria: Gingival healthy group, GI=0, with PD < 3mm and no attachment loss or clinical sign of BOP, no erythema or supuration. Gingivitis group, GI≥ 1, no relevant clinical attachment loss, PD < 3 mm, bleeding on probing and the presence of either swelling or redness. Furthermore, based on GI values, gingivitis group was subdivided into the mild (GI=1), moderate (GI=2) and severe (GI=3) gingivitis subgroups.

Finally, in order to make a relevant analysis of the putative relationship between IL-18, IDDM and periodontal status, the global 60 young individuals were assigned into 8 sub groups as follows:

Control:

- Gr. 1= healthy (GI= 0), n=10 (subjects), s=80 (number of GCF collected samples);
- Gr. 2 = mild gingivitis (GI=1), n=11, s = 88;
- Gr. 3 = moderate gingivitis (GI=2), n=8, s=64;
- Gr. 4 = severe gingivitis (GI=3), n=1, s=8;

IDDM group:

- ✓ Gr. 5 = healthy (GI= 0), n=3, s=24;
- ✓ Gr. 6 = mild gingivitis (GI=1), n=5, s=40;
- ✓ Gr. 7 = moderate gingivitis (GI=2), n=16, s=128;
- ✓ Gr. 8 = severe gingivitis (GI=3), n=6, s=48.

○ *Procedures for site selection and sample collection*

In order to assess the cytokine levels GCF we used samples collected using paper strips. For all the selected participants, the periodontal examination and GCF collection were performed in the dental office. The sample collection was performed in mesial sites of central incisors (I) and the first permanent molars (M, maxillary and mandibular) of each individual, resulting in a total of 8 strips per patient. Sample collection was achieved before clinical evaluation to avoid any contamination of the strips with blood released during the periodontal evaluation.

The area was isolated with cotton rolls and gently air-dried to remove possible saliva contamination. Gingival fluid was collected through inserting standard paper strips (Periopaper, Oraflow Inc., NY, USA) into the sulcus for 30s. Strips that were contaminated with blood were discarded. The GCF volume was recorded with a calibrated device (Periotron 8000, Proflow Inc., Amityville, NY, USA), the readings being subsequently converted to accurate volumes by reference to a standard curve. Strips were placed into specific vials containing 100 microliters of phosphate saline buffer and stored at -70°C until analysis for IL-18. Calculation of the concentration in each sample was performed by dividing the amount of substances by the volume of the sample (ng/mL).

○ *IL-18 assay*

The GCF samples were assayed for IL-18 levels using Human IL-18 ELISA kit, according to the manufacturer's instructions. In brief, the technique is based on anti-IL-18

monoclonal coating antibody that is adsorbed onto micro-wells. IL-18 present in the sample or standard binds to antibodies adsorbed to the micro-wells. Subsequent to the addition of a biotin conjugated monoclonal anti-IL-18 antibody, it binds to IL-18 captured by the first antibody. Streptavidin-HRP is added and binds to the biotin conjugated anti-IL-18. Finally, a colored product is formed, the intensity being directly proportional to the amount of IL-18 present in the sample. Absorbance of each well is read on ELISA reader using 450. nm as primary wavelength. The concentration of IL-18 in the analyzed samples was estimated using the standard curve.

- ***Statistical analysis***

Statistical analysis was performed using a software package. The assess the data distribution normality, the Kolmogorov-Smirnov test was used. The parametric tests were needed to compare the means of IL-18 values in different groups. Statistical analysis was performed using One Way ANOVA completed with Holm-Sidak method, with confidence level of 95% ($p < 0.05$). Finally, the use of Pearson's correlation was involved to observe any existing correlation between the IL-18 GCF amounts and clinical parameters. Analysis considered the $p<0.05$ values statistically significant. Mean ± SD was used to express results per groups.

II.2.3. RESULTS

Table II.2.2 display the distribution of the patients in all groups according to the gingival health status. It was seen that 10 subjects in the control group showed no periodontal damage (GI = 0), significant difference compared to IDDM group of children, whereas only three individuals displayed absence of periodontal modification (33.33 % vs 10 %).

Table II.2.2. *Periodontal diagnosis (patients and percentage)*

Periodontal diagnosis		Control (n=30)	DID (n=30)
Absence of periodontal disease (GI=0)		10 (33.33%)	3 (10%)
Gingivitis (GI≥1)	GI=1	11 (36.66%)	5 (16.66%)
	GI=2	8 (26.66%)	16 (53.33%)
	GI=3	1 (3.33%)	6 (20%)

The majority of children in the two studied groups exhibited different degrees of bacterial gingivitis, as follows:

- mild gingivitis (GI=1) was recorded in 11 subjects of the control group and 5 subjects of the IDDM batch (36.66% vs 16.66%);
- moderate gingivitis (GI=2) was recorded in 8 subjects of the control group and 16 subjects of the IDDM group (26.66% vs 53. 33%);
- severe gingivitis (GI=3) presented in only 1 children from the control group and 6 subjects of the diabetic group (3.33% vs 20%).

Is to be noted that while for most children in the control group gingival inflammation was mild or absent, in the case of diabetic young subjects, moderate to severe gingival inflammation were dominating. No case of chronic or aggressive

periodontitis have been diagnosed in the studied groups, as no attachment loss greater than 2 mm (CAL > 2 mm) nor PD > 3 mm were detected.

- ***Clinical indices***

The average values for clinical indices in the enrolled subjects are found in Table II.2.3.

There are no statistically significant differences in PI, PD and CAL values between control groups and the corresponding IDD groups ($p > 0.05$), (5 vs 1, 2 vs 6, 7 vs 3 and 4 vs 8).

When comparing control groups and their corresponding IDD subjects, statistically significant differences were revealed for BOP and GI indexes, more relevant for the inflammatory status ($p < 0.05$) (Table II.2.3).

Table II.2.3. *Clinical indices (t-test analysis)*

Parameters	Control	IDD	P value
PI (mean±SD)	Gr. 1 (GI=0) Gr. 2 (GI=1) Gr. 3 (GI=2) Gr. 4 (GI=3)	Gr. 5 (GI=0) Gr. 6 (GI=1) Gr. 7 (GI=2) Gr. 8 (GI=3)	Gr 1 vs 5, p=0.431 Gr 2 vs 6, p=0.199 Gr 3 vs 7, p=0.075 Gr 4 vs 8, p=0.802
GI (mean±SD)	Gr. 1 (GI=0) Gr. 2 (GI=1) Gr. 3 (GI=2) Gr. 4 (GI=3)	Gr. 5 (GI=0) Gr. 6 (GI=1) Gr. 7 (GI=2) Gr. 8 (GI=3)	Gr 1 vs 5, p=0.445 Gr 2 vs 6, p=0.218 Gr 3 vs 7, p=0.025* Gr 4 vs 8, p=0.023*
BOP (mean±SD)	Gr. 1 (GI=0) Gr. 2 (GI=1) Gr. 3 (GI=2) Gr. 4 (GI=3)	Gr. 5 (GI=0) Gr. 6 (GI=1) Gr. 7 (GI=2) Gr. 8 (GI=3)	Gr 1 vs 5, p=0.073 Gr 2 vs 6, p=0.195 Gr 3 vs 7, p<0.01* Gr 4 vs 8, p=0.025*
PD < 3mm (mean±SD)	Gr. 1 (GI=0) Gr. 2 (GI=1) Gr. 3 (GI=2) Gr. 4 (GI=3)	Gr. 5 (GI=0) Gr. 6 (GI=1) Gr. 7 (GI=2) Gr. 8 (GI=3)	Gr 1 vs 5, p=0.308 Gr 2 vs 6, p=0.719 Gr 3 vs 7, p=0.151 Gr 4 vs 8, p=0.397
CAL < 2mm (mean±SD)	Gr. 1 (GI=0) Gr. 2 (GI=1) Gr. 3 (GI=2) Gr. 4 (GI=3)	Gr. 5 (GI=0) Gr. 6 (GI=1) Gr. 7 (GI=2) Gr. 8 (GI=3)	Gr 1 vs 5, p=1.000 Gr 2 vs 6, p=1.000 Gr 3 vs 7, p=0.355 Gr 4 vs 8, p=0.183

*p < 0.05, statistically significant

In the context of the periodontal disorders initiated by the accumulation of bacterial plaque, the inflammatory reaction starts early into the childhood and reflects the major significance of bacterial impact on the host, in a systemic context. For most children, the inflammatory process of the gums remains superficial, at the clinical stage of gingivitis. In some cases, however, the balance between the microbial load and the host response is disrupted and leads to a destruction of the support tissues of the teeth, which can sometimes result in the loss of dental units.

Based on the large evidence in the literature which showed an increased incidence and severity of the periodontitis in subjects affected by diabetes, our study tried to shed a new light upon this, by investigating the bidirectional relationship between the two disorders through IL-18.

Table II.2.4. *Descriptive statistics of studied patients showing mean, standards deviation, mean and range for the PI, GI, BOP, CAL, PD and IL-18 levels in GCF*

	Study group	IL-18 (Mean ± SD)(pg/ml)	PI (Mean ± SD)	GI (Mean ± SD)	BOP (Mean ± SD) %	PD (Mean ± SD) (mm)	CAL (Mean ± SD) (mm)
control	Goup 1 GI=0	I_1=8.3±0.2 M_1=3.2±0.1	0.5±0.4	0.3±0.4	0	1.0±0.9	0
	Group 2 GI=1	I_2=17.4±0.9 M_2=12.5±0.3	0.8±0.3	0.6±0.4	0.7±0.2	1.8±0.4	0
	Goup 3 GI=2	I_3=19.5±0.8 M_3=12.8±0.3	1.5±0.3	1.2±0.6	16.2±5.4	2.0±0.7	0.5±0.8
	Goup 4 GI=3	I_4=25.7±1.3 M_4=15.9±0.4	1.8±0.7	2.1±0.9	28.6±4.3	2.4±0.3	0.7±0.9
DID	Group 5 GI=0	I_5=32.6±1.7* M_5=24.2±1.4*	0.7±0.2	0.5±0.3	0.5±0.9	1.6±0.6	0
	Group 6 GI=1	I_6=35.7±2.6* M_6=26.8±3.3*	1.0±0.2	0.9±0.5	0.9±0.4	1.9±0.7	0
	Group 7 GI=2	I_7=44.4±5.3* M_7=33.8±4.1*	1.8±0.4	1.9±0.7	31.9±6.2	2.3±0.3	0.8±0.7
	Group 8 GI=3	I_8=68.9±8.2* M_8=42.5±6.3*	2.0±0.7	2.8±0.2	47.4±5.5	2.5±0.1	1.2±0.3

I = incisor; M = molar; IL-18 = interleukin 18; PI = plaque index; GI = gingival index;
BOP = bleeding on probing; PD = pocket depth; CAL=clinical attachment loss
*statistically significant ($p < 0.05$) as compared to control groups (1-4)

- ***Interleukin-18***

The descriptive data for concentrations of IL-18 in GCF in all the 1-8 subgroups are presented in the Table II.2.5.

The results indicated the mean IL-18 concentration in GCF was highest in group 8- diabetic subjects with severe gingivitis and lowest in group 1- control group and gingivitis-free.

Between these uttermost values, mean IL-18 levels in GCF were found, as descending trend, in IDD children with moderate gingivitis, in IDD subjects with mild gingivitis, in IDD and no gingival inflammation, in control group with severe gingivitis, in control group with moderate gingivitis and in control group with mild gingivitis. These data highlighted the clear conclusion that the effects of diabetes on GCF IL-18 levels is still to be debated. Differences in parameters to define periodontal status, management of various insulin types and others such as the protocol to assess interleukins values should account for these results.

Table II.2.5. *Pearson's correlation coefficient test comparing GCF IL-18 and other variables for incisors (I) and molars (M)*

Groups	IL-18 and PI (I/M)	IL-18 and GI (I/M)	IL-18 and BOP (I/M)	IL-18 and PD (I/M)	IL-18 and CAL (I/M)
Group 1	0.925/0.843	0.924/0.811	0.867/0.735	0.996/0.984	0.841/0.719
Group 2	0.961/0.979	0.969/0.920	0.899/0.969	0.843/0.961	0.827/0.811
Group 3	0.979/0.961	0.920/0.969	0.925/0.979	0.867/0.920	0.985/0.719
Group 4	0.841/0.956	0.956*/0.843*	0.989*/0.827*	0.827/0.735	0.757/0.811
Group 5	0.935/0.949	0.991*/0.996*	0.960*/0.977*	0.953/0.957	0.959/0.976
Group 6	0.773/0.973	0.872*/0.972*	0.891*/0.991*	0.883/0.971	0.851/0.951
Group 7	0.960/0.984	0.953*/0.978*	0.959*/0.983*	0.978/0.953	0.984/1.000
Group 8	0.953/0.978	0.972*/0.827*	0.973*/0.735*	0.953/0.843	0.960/0.867

*statistically significant

Our results pointed out that the mean values of the gingival fluid IL-18 in group 5-IDDM without clinical expression of gingivitis (GI=0)**,** were more elevated than those recorded in the control group regardless of mild, moderate and even severe gingivitis (group 2, group 3 and group 4).

One Way ANOVA completed with Holm-Sidak method revealed that the differences were statistically significant between group 1 *vs* 5 ($p<0.001$), 2 *vs* 6 ($p<0.001$), 3 *vs* 7 ($p<0.001$), 4 *vs* 8 ($p<0.001$), 5 *vs* 2 ($p<0.001$), 5 *vs* 3 ($p<0.001$), 5 *vs* 4 ($p=0.021$), for both incisors and molars.

Pearson's correlation (Table II.2.5.) exhibited the positively correlation between the GCF IL-18 level and GI as well as BOP (and not with PI, CAL, PD) in groups 4, 5, 6, 7, 8 for both incisors and molars.

II.2.4. DISCUSSIONS

Following the microbial attack, the body responds by releasing various cytokines. Their type and concentration control the nature and intensity of the inflammatory response. Of these, IL-18, a multifunctional pro-inflammatory cytokine caught our attention. IL-18 belongs to the IL-1 family and many studies found positive correlations between the IL-18 levels in the serum, GCF or the gingival tissue, and the clinical indexes of periodontitis, i.e. GI, PD, BOP and CAL.

The most promising results for the correlation of GCF composition, in both quantitative and qualitative terms, with the periodontal inflammatory status have been obtained by the detection and quantification of inflammatory mediators (Kurdukar, 2015). These mediators include cytokines (interleukins, tumor necrosis factors), prostaglandins (PGE) and matrix-metalloproteinases (MMPs), playing different roles in promoting and fueling the inflammatory reaction. Secreted by a large number of cells, including immune cells like neutrophlis, macrophages or lymphocytes and non-immune cells such as fibroblasts and epithelial cells these mediators act as signaling elements for the activation of immune cells that are aimed to resolve a bacterial challenge. In addition, they promote and drive the inflammatory reaction, causing tissue changes by enzyme activity (Preshaw, 2011).

The cytokine group of inflammation mediators, studied within the GCF includes interleukins and tumor necrosis factors. The interleukin (IL) family is extremely diverse and play crucial role in the onset and progression of the periodontal inflammatory response. Interleukin-1β (IL-1β) is a major cytokine, involved in the triggering of the inflammatory chain reaction and stimulating the secretion of other pro-inflammatory mediators. In periodontal pathology, IL-1β enhances the inflammatory reaction and accelerates bone resorption (Honig, 1989). GCF levels of IL-1β are more elevated than average in patients with gingival and periodontal inflammation and they correlate to the periodontal clinical status of the diseased-patients, in terms of severity (Gomes, 2015). Interleukin-1α (IL-1α) also has an important impact on bone resorption and the fueling of the periodontal inflammatory reaction (Dinarello, 2009). Its GCF levels were also found to be increased in periodontal patients (with alveolar bone loss), probably as a consequence of its release into the damaged tissues by affected cells (Rasmussen, 2000). Both IL-1β and IL-1α can be considered powerful bone resorption driving mediators and their presence in GCF in increased levels sustains the possible use of GCF as an assessment tool of periodontal disease risk, evolution and prognosis.

Other interleukin involved in the regulation of the periodontal inflammatory reaction, such as IL-6, IL-1Ra and IL-18 have also been found in increased levels in GCF

samples originating from periodontal patients, these levels being correlated to the clinical periodontal status of the subjects (Buduneli, 2011). The tumor necrosis factor alpha (TNF-α) is another member of the cytokine group, acting as a key mediator in periodontal pathogenesis, by stimulating neutrophil cell, MMPs and bone resorption activity and by limiting the reparatory potential of fibroblast (Graves, 2003). Increased GCF levels of TNF-α are also found in periodontal patients and they may increase further as the inflammatory reaction progresses (Gokul, 2012).

There was found an association between the raise of IL-18 concentrations and the increase of the risk to develop type 2 diabetes, by age, body mass index, systolic blood pressure and physical activity. The study was performed on adult subjects after adjustment for classic risk factors.

IL-18 is responsible for the initiation and propagation of periodontal destruction. There are studies indicating that IL-18 induces the synthesis of MMP 9 and IL-1β, both with pro-inflammatory effect in tissue degradation (Sapna, 2014).

Since these events also occur in chronic periodontal inflammation, it seems worthwhile to evaluate IL-18 levels in normal and diseased periodontium.

Our study included eight subgroups, which resulted from subdividing each of the two main groups (control and IDDM) based on the GI value, i.e. two healthy subgroups (GI=0), two mild gingivitis subgroups (GI=1), two moderate gingivitis subgroups (GI=2) and two severe gingivitis subgroups (GI=3).

Regarding the values of the clinical indices, our results indicated the lack of significant values PI in the diabetic batch as compared to the control group. This is in agreement with some authors (Schenkein, 2013) which also reported that PI was not significantly different between the control group and the IDDM, and in disagreement with others (Delima, 2003), which found that the values of PI in diabetic patients (especially those with a poor metabolic control) were significantly higher ($p<0.0001$) as compared to healthy patients.

Our results showed statistically significant differences between the control and IDDM groups for GI and BOP, being in agreement with the extensive literature data.

The investigations performed in our groups of young subjects clearly demonstrated the increase of IL-18 concentration in the GCF proportionally with the progression of the gingival inflammation.

The IL -18 values increased with the GI and BOP values, in both the diabetic and control groups, which is in agreement with other studies performed on diabetes-free adults. In addition, this increase of the IL-18 in the GCF was strongly associated with the presence of diabetes in children.

The IL-18 values were 2.7-2.8 times as average higher in the IDD group as compared with the control group, both with severe inflammation. The same is true for corresponding subgroups as well (IDDM *vs* control). These results could not be compared with literature data since we could not find any studies regarding the assessment of IL-18 in the GCF of children with IDDM. As mentioned above, the IL-18 was evaluated in the serum/GCF of adults with periodontitis but without diabetes.

While investigating IL-18 in the serum of subjects with and without periodontal impairment, it was found that serum IL-18 levels increased with the severity of the periodontal disease. Moreover, the study has demonstrated positive correlation among IL-18 levels in serum and GI, PD and CAL in chronic periodontitis.

Markedly increased levels of IL-18 were found in serum of juvenile idiopathic arthritis (JIA) patients with incipient attachment loss. Part of the authors of the same group reported a higher elastase activity associated with lower IL-18 in GCF from juvenile systemic lupus (JSLE) patients, the IL-18 values being in the GCF, 33 ± 38 pg/mL (JSLE)

vs. 52 ± 14 pg/mL (control), but higher in the serum, 453 ± 302 pg/mL (JSLE) vs. 315 ± 61 pg/mL (control). The average age of the adolescent patients in the study was 15.6 ± 2.7 years old.

We presented the above data to be able to compare the IL-18 values from our studied groups with published data regarding children and adolescents (which are scarce and conflicting).

Moreover, the statistical differences between the IL-18 volumes from the IDDM group and those from the control group are much higher as compared with the differences between the clinical indexes values (GI, BOP). In contrast with some important studies (Leppilahti, 2014), we found no significant differences between the studied groups regarding the values of the PI and CAL clinical indexes. The present study also shows statistical correlations between IL-18 and the clinical indexes (GI, SBI) in some subgroups, in agreement with others.

As shown, the GCF is a valuable and smooth interface that can be used for the assessment of the periodontal status. Nevertheless, its small quantity in periodontal healthy patients makes it difficult to sample in order to provide control group samples for a study. The sampling methods vary from researcher to researcher and include using intra-sulcular absorbent twisted threads, aspiration of the fluid with micro-pipettes or washings of the gingival sulcus (Egelberg, 1973). However, the most commonly used method of GCF sampling is the usage of absorbent intra-sulcular paper strips. These paper strips are inserted into the gingival sulcus and kept there for about 30 seconds, until they become saturated with the fluid. Afterwards, the paper strips are disposed into small plastic containers, usually filled with phosphate-buffered saline and refrigerated until further use. Although may seem simple, careful handling of the paper strips needs to be adopted, so as not to contaminate them with traces of saliva or blood.

This is more difficult when the gingival tissues are inflamed and bleed easily, or when the sampling is done from the vicinity of the salivary glands' excretory canals. Additional isolation measures such as cotton rolls and saliva suction could be used. After GCF sampling, the amount of fluid on each paper strip should be determined. This is done by using a specially designed device, the Periotron (Harco Electronics, Winnipeg, Manitoba, Canada) (Suppipat, 1977). The device works on a simple principle: the more GCF the paper strips contains the more electricity it can conduct. It is thought that a paper strip of 1.5 mm wide can absorb about 0.1 µl of GCF (Wassall, 2016).

The composition of GCF is a reliable indicator of the periodontal status, in health and inflammation, in systemic integrity and disease and at cellular and molecular level. The tests used for GCF assessment are numerous and target specifically aimed elements: fluorometry (for MMPs), enzyme-linked immunosorbent assay (for other enzyme and cytokines), radioimmunoassay (for cyclooxygenase and procollagen), chromatography (for timidazole) and immun0-enzymatic assay (for acute-phase proteins). Some commercial test kits have been developed for the simple and fast detection of GCF components, as diagnosis-aid tools or screening instruments for periodontal disease. These include Periocheck (for proteinases), Prognostik and Biolise (for elastases), MMP dipstick (for MMPs), TOPAS (for bacterial toxins and proteases) and Pocket watch (for AST) (Mantyla, 2003; Oswal, 2010). Nevertheless, these GCF diagnosis tests should be used only as complementary diagnosis tools alongside with a comprehensive oral and periodontal examination, periodontal probing and radiologic exam. Their usage is recommended during screening and risk-assessment programs for periodontal disease (Lamster, 2007).

Furthermore, IL-18 could serve as a marker of inflammation at the preclinical stage of the periodontal impairment, in the context of juvenile insulin-dependent diabetes. Hence, the concentration of IL-18 in GCF increases with the progression of periodontal

inflammation, and is extensively associated to the presence of diabetic systemic alteration (mean GCF IL-18 levels being elevated in all diabetic individuals' subgroups, as compared to non-diabetics).

II.2.5. CONCLUSIONS

- The present results are part of the first study targeting investigation of IL-18 in the GCF of children with diabetes.
- Our results point out that IL-18 level in GCF grows with the severity of the gingival inflammation, but is also highly elevated in the systemic context of diabetic impairment.
- GCF expresses a high sensitivity towards the changes occurring within the periodontal structures during the onset, evolution or remission of periodontal inflammation. GCF also reflects the important connections that exist between the periodontal pathology and certain systemic conditions, comprising the concept of "periodontal medicine".
- GCF allows comprehensive and extensive research in the field of periodontology and has proven to be a valuable asset for numerous studies. Along with other important clinical periodontal parameters, the GCF offers a smooth interface towards the pathogenic processes that govern periodontal disease, as well as enabling solutions for the disease's therapy and recovery.

II.3. LABORATORY INVESTIGATIONS FOR ASSESSMENT OF GENERAL AND ORAL HOMEOSTASIS – A REAPPRAISAL FROM BIOCHEMICAL AND PHARMACOLOGICAL VIEWPOINT

II.3.1. Evaluation of the soft and hard oral tissues during pathologic context

II.3.1. State of the art

Oral tissues are continuously exposed to damage triggered by the mechanical effort of feeding or due to germs, mostly bacteria. In healthy gingiva tissue remodelling and a harmony between bacteria and innate immune cells are maintained. However, excess of bacteria biofilm triggers inflammatory status, recruiting more immune cells, mainly neutrophils to the gingiva.

These leukocytes create a barrier for bacteria to reach inside tissues, and whenever insufficient, bacteria thrive causing more inflammation that has been associated with systemic effects on other conditions such as atherosclerosis or diabetes. Neutrophils persistence can promote a chronic inflammatory state that leads to periodontitis, a condition that determines damage of the bone-supporting tissues, as well.

Gingival overgrowth, also known as gingival hyperplasia or hypertrophy, is an abnormal enlargement of gingival tissue. There are several causes of gingival enlargement and they can be grouped into four categories: 1) inflammatory gingival enlargement, 2) drug - induced gingival enlargement, 3) hereditary gingival fibromatosis, and 4) systemic causes of gingival enlargement.

Certain medications can lead to gingival overgrowth in patients having systemic involvement such as in epilepsy or organ transplant. In contrast to inflammatory gingival hypertrophy, typically the gum tissues in such cases are firm, non - tender, pale pink in color, and do not bleed easily. In severe cases, the gingiva may completely cover the crowns of the teeth causing difficulties in chewing, inflammation, as well as tooth eruption and alignment disturbance. Drug-induced gingival overgrowth may resolve either partially or completely when the medication is discontinued. If the medication cannot cessate, surgical removal of the excessive gingiva (gingivectomy) could be the solution, but the condition will likely reoccur (Luchian et al, 2016). Clinical and cell culture studies suggest that the mechanism of gingival overgrowth is a result of the interaction between the drug and its metabolites with susceptible gingival fibroblasts.

Gingival hyperplasia occurs mainly as a consequence of the treatment with certain antiepileptic, immunosuppressant or antihypertensive drugs. The excess gingival tissue affects oral health as well as esthetics. Oral hygiene is compromised by gingival overgrowth, which can have a negative impact on the systemic health of the patients.

Cyclosporine A (CsA) is an immunosuppressant widely used to prevent transplant rejection, but also for the treatment of certain autoimmune diseases such as bullous

pemphigoid, psoriasis and rheumatoid arthritis. CsA induced gingival hyperplasia was first described in 1983 by Rateitschak - Plüss and is estimated to occur in about 30% of the patients following this medication (Rateitschak - Plüss et al, 1983; Mishra et al, 2011).

In medical practice, calcium (Ca^{2+}) channel blockers are frequently used in cardiovascular disease management. Most classes of Ca^{2+} channel blockers were proved to be involved in gum overgrowth (Şurlin et al, 2009), Nifedipine being the most common agent causing Ca^{2+} channel blocker-induced gingival hyperplasia.

The interactions between CsA, Nifedipine and gingival fibroblasts may have an essential contribution to gingival hyperplasia. Variable direct behavioral responses of fibroblasts in the presence of CsA and Nifedipine include a modified metabolic activity.

As previously described (Silvestri, 2000), cyclosporine A inhibits this permeability pore, thus inhibiting cation transport through mitochondria permeability transition pore (MTP) and Ca^{2+} cannot leave mitochondria using this pathway or the outward flow rate is very low. Several data suggest that apoptosis plays an important role in gingival overgrowth controlling and that involves a cascade of biochemical steps that require an increase of intracellular Ca^{2+}. Earlier studies showed that fibroblast apoptosis is decreased in gingival overgrowth, one of the best described mechanisms in apoptosis induction being represented by the increases cytosolic Ca^{2+} in the investigated cells (Mattson et al, 2003).

Being also called *programmed cell death* (PCD), apoptosis represents an active process, involving a predetermined program of molecular interactions. It has an important role in controlling the normal development of the embryo in certain diseases such as cancer, degenerative diseases or aging.

The term apoptosis was introduced in biology by Keer in 1972, in order to designate a particular form of cell death (Keer et al., 1972). Being a fundamental physiological process of pluricellular organ life, apoptosis is equally a suicidal mode of cellular response to an external chemical or physical aggression nature.

The contributing papers to this direction are:

1. Caraiane A, **Toma Vasilica**, Raftu G, Debita M, Dimofte AR, Iordache C. Aesthetic Rehabilitation of the Teeth using Single Fixed Prostheses. *Rev Chim*. 2019; 70(2): 714 - 717. (IF 2017= 1, 412)
2. Goriuc A, Foia LG, Minea B, Luchian AI, Surdu AE, **Toma Vasilica**, Costuleanu M, Martu I. Drug - induced gingival hyperplasia – experimental model. *Rom J Morphol Embryol*. 2017; 58(4): 1371 - 1376. (IF = 0, 912)
3. Cergizan D, Branasco T, Brujbu IC, **Toma Vasilica,** Iordache C. Restoration the Dento-facial aesthetic balance, a target of dental therapy, regardless the patient's age. *Rev Chim*. 2017; 68(10): 2358 - 2362. (IF= 1, 412)
4. Goriuc A, Minea B, Foia L, Costuleanu M, Jipu R, **Toma Vasilica,** Vlad C, Luchian I, Martu I. The role of mitochondrial calcium overload in cyclosporine A - induced gingival hyperplasia. *Romanian Journal of Oral Rehabilitation*, 2017; 9(3): 55 - 61.

II.3.1.2. MATERIALS AND METHODS

Experimental model of drug- induced gingival hyperplasia

The main goal of our research consisted of the effects of CsA and Nifedipine on the morphology and viability of cultivated gingival fibroblasts obtained from rats.

- *Normal and CsA - /Nifedipine - treated fibroblast cultures achievement*

Gingival fibroblasts were obtained from male rats gingiva by explant technique. After harvesting, gingival samples were minced, washed six times with phosphate - buffered saline (PBS) and then placed into Petri dishes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 584 mg/L L - Glutamine, 4500 mg/L Glucose, 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin antibiotic mix, and 1% Amphotericin B (Figure II.3.1.2.1).

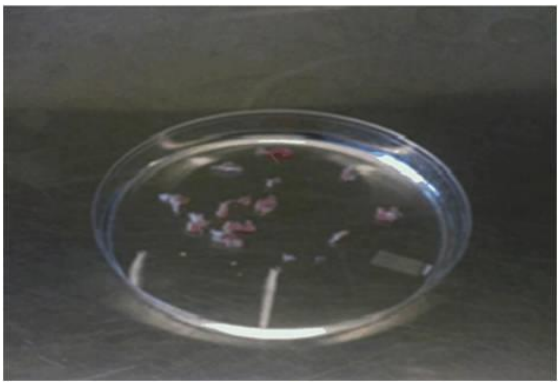

Figure II.3.1.1. *Minced rat gingival samples*

The samples were incubated for 2–7 days at 37°C and 5% CO_2. The gingiva was removed and the fibroblasts that were attached to the walls were left to further multiply in fresh medium at 37°C and 5% CO_2 for another 48–72 hours. After two more passages, the cells were divided into three groups and placed in the same culture medium mentioned above.

Assessment of CsA and Nifedipine effects

- Group I - the control group – no treatment.
- Group II - was treated with 1 μg/mL (1 μM) CsA,
- Group III - treated with 5 μg/mL (3 mM) Nifedipine.

All three groups were then incubated at 37°C and 5% CO_2, until they became fully confluent. During the incubation, the cells were photographed at 7, 14 and 30 days, with a phase contrast Nikon Eclipse TE300 microscope and its software. The photographs were taken at 40×, 100× and 200× magnification.

The effect of the drugs on gingival fibroblast viability was assessed by immunofluorescence (flow cytometry) with a FACS (fluorescence - activated cell sorting) Calibur device and its software. The used acquisition settings were FL1 623 V, FL2 505 V, 10 000 events and 488 nm laser. Data analysis was performed by using FlowJo® 7.6.1 software.

The preparation of drug - treated cells for flow cytometry involved trypsinization with Trypsin ethylene - diamine – tetra-acetic acid (EDTA), washing by repeated suspension in PBS and centrifugation at 300×g for 5 minutes, and, finally, resuspension in 1mL of culture medium. The cells were then counted (approximately 10^6/mL) and equally divided into several test tubes. One test tube was assigned to be the control while in the others 5 μL/mL (2 μM) of Calcein and 5 μL/mL (80 mM) of $CoCl_2$ were added. The test tubes were then incubated at 37°C and 5% CO_2 for 20 minutes.

- ***Cyclosporine A - induced gingival hyperplasia: the role of mitochondrial calcium overload***

Related to the hyperplasia effect mediated by various drugs, we also investigated the sensitivity of normal gingival fibroblasts compared to those treated with CsA in culture medium, toward apoptosis induced by Ca^{2+} overload.

We evaluated the effect of ionomycin and A23187 ionophore upon normal gingival fibroblasts and also on fibroblasts treated with CsA, using flow cytometry methods. Initiation phase of apoptosis, induced by Ca_2^+ overload is represented by opening of mitochondrial permeability transition pore (MTP).

Gingival fibroblasts were obtained from male rats, 150 – 170 g weight, by gingival explants and grown up in specific culture medium supplemented with fetal bovine serum and antibiotics. After we obtained fibroblasts we divided them into two groups: one control group that received no treatment, and one group treated with CsA (1μg/ml).

The protocol consisted in normal and treated fibroblasts trypsinization, their flush through centrifugation at 300 x g for 5 minutes, with subsequent resuspension in 1 ml culture medium. Cells were counted (about 1, 000, 000/ml) and were equally divided in tubes. A tube was depicted as control group, while in other tubes we have added calcein 5μl/ml (2μM concentration) and 5μl/ml of $CoCl_2$ (concentration of 80mM) and allowed for 20 minutes incubation, at 37°C and 5% CO_2. In the other two tubes, we added 100μl/ml $CaCl_2$ (1μM) and calcium ionophore - like ionomycin (1 mM) and A23187 (10 μM) for 24 h.

After 14 days, the mitochondrial transient permeability pore (MTP) function was monitored by flow cytometry using a Calibur type FACS and related software. The settings used for the acquisition were: FL1 623 V, FL2 505 V, 10, 000 events, 488 nm laser. The data was processed with FlowJo 7.6.1 software.

II.3.1.3. RESULTS

- ***Experimental model of drug-induced gingival hyperplasia***

Regarding our investigation upon morphological modifications in CsA - and Nifedipine - treated gingival fibroblasts, no major morphological modifications of gingival fibroblasts following their isolation from rat gingiva could be observed. The CsA and Nifedipine administrations, however, induced morphological changes in gingival fibroblasts.

The cell proliferation increased with drug exposure time, the morphological shift including altered shapes and the presence of cytosol drug accumulations (Figure II.3.1.2 and Figure II.3.1.3).

Hematoxylin and Eosin staining (Figure II.3.1.4) showed the loss of the star shape in fibroblasts treated with CsA and Nifedipine for more than 14 days.

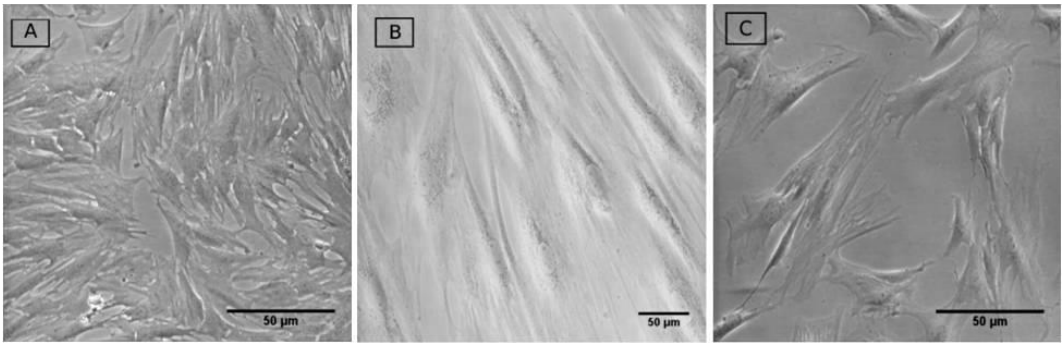

Figure II.3.1.2. *Gingival fibroblasts after seven days of incubation (×100; Scale bar = 50 µm): (A) Normal; (B) CsA treated 1 µM); (C) Nifedipine treated (1 mM). CsA: Cyclosporine A.*

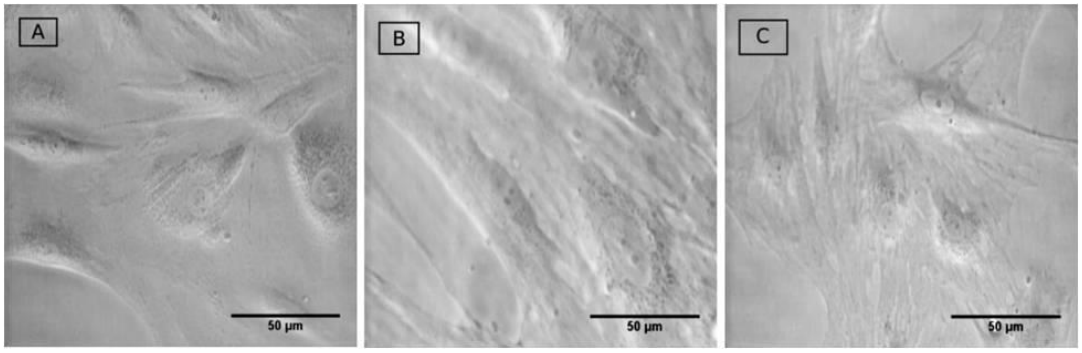

Figure II.3.1.3. *Gingival fibroblasts after 30 days of incubation (×100; Scale bar = 50 µm): (A) Normal; (B) CsA treated (1 µM); (C) Nifedipine treated (1 mM).*

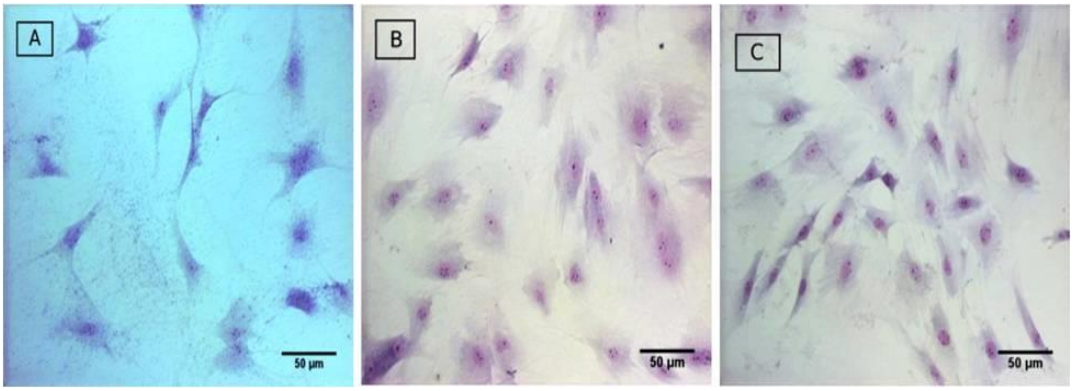

Figure II.3.1.4. *Hematoxylin and Eosin staining of gingival fibroblasts (×100; Scale bar = 50 µm): (A) Normal; (B) CsA treated (1 µM); (C) Nifedipine treated (1 mM). CsA: Cyclosporine A.*

- *Fibroblast viability*

For the study of the effects exhibited by CsA and Nifedipine on the viability of gingival fibroblasts we used Calcein AM (ester of Calcein with acetoxymethyl), a non - fluorescent molecule that passively diffuses through the membrane of living cells and accumulates in cytosol compartments including the mitochondria (Figure II.3.1.5).

As a consequence, Calcein AM is a useful tool in assessing cell membrane integrity, and has a very low toxicity. Inside the cells, the acetoxymethyl esters are hydrolyzed - this generates negatively polarized Calcein, intensely fluorescent (green emission) and is retained in the cytoplasmic compartments of living cells. To reduce the intensity of the cytoplasmic fluorescence, the cells were incubated with $CoCl_2$.

When we evaluated the fluorescence histograms we noticed a decreased viability of Nifedipine (1 mM) - treated cells (Figure II.3.1.6), while CsA (1μM) - treated cells, however, showed a slightly increased viability (Figure II.3.1.7). This is most probably due to a mechanism of blocking the mitochondrial permeability transition pore, which is known to be involved in triggering the first step of apoptosis.

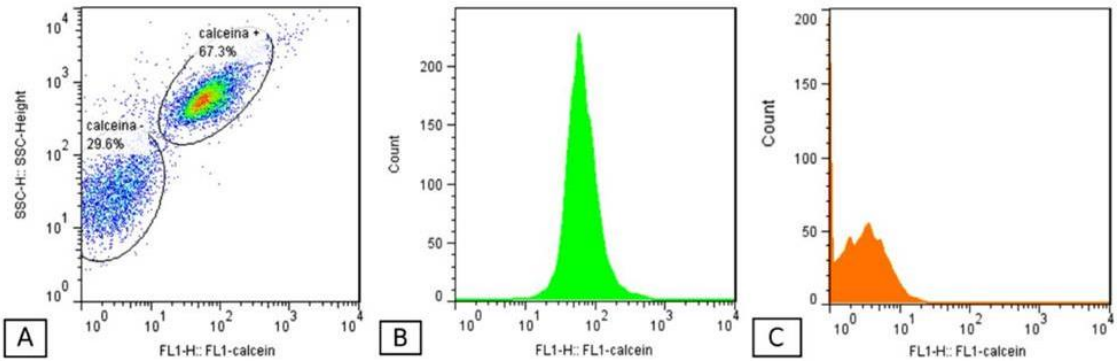

Figure II.3.1.5. *Representative FACS image for the Calcein loading of normal gingival fibroblasts: (A) FACS image;(B and C) Fluorescence histograms. FACS: Fluorescence - activated cell sorting*

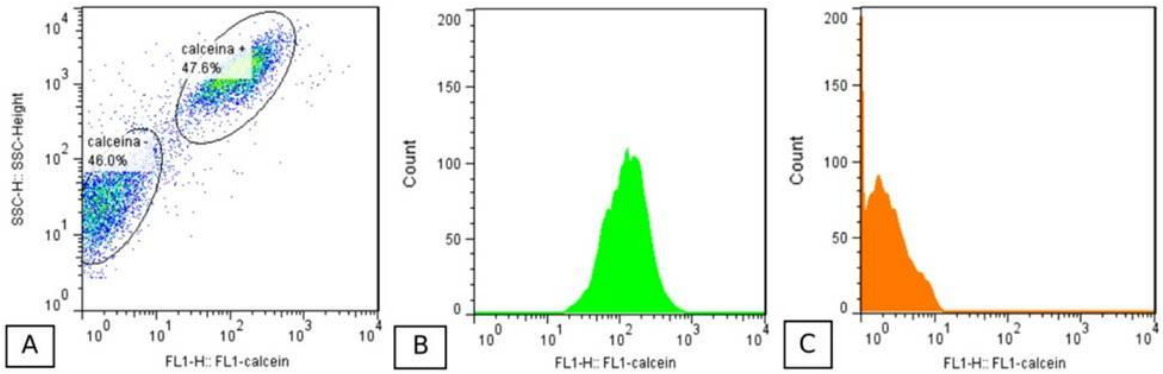

Figure II.3.1.6. *Representative FACS image for the Calcein loading of Nifedipine - treated gingival fibroblasts: (A) FACS image; (B and C) Fluorescence histograms. FACS: Fluorescence - activated cell sorting*

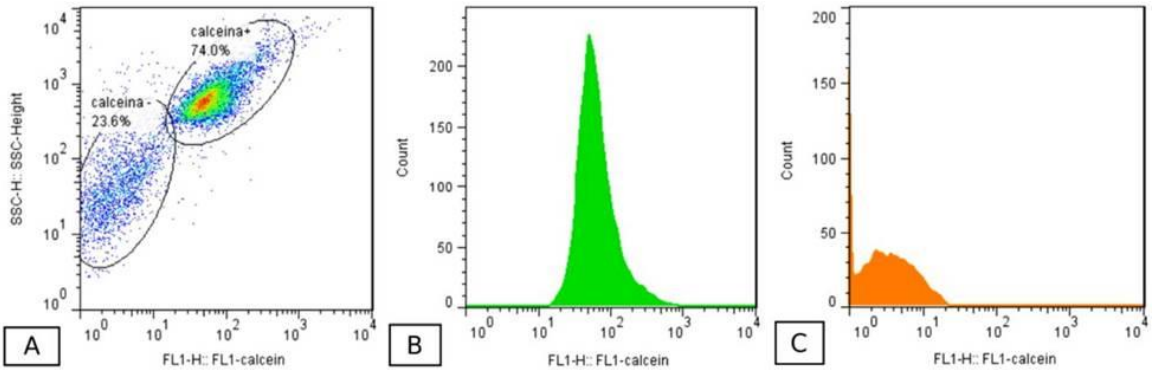

Figure II.3.1.7. *Representative FACS image for the Calcein loading of CsA - treated gingival fibroblasts: (A) FACS image; (B and C) Fluorescence histograms. FACS: Fluorescence - activated cell sorting; CsA: Cyclosporine A*

- ***Cyclosporine A - induced gingival hyperplasia the role of mitochondrial calcium overload***

After cells loading with calcein AM, intracellular esterases cleave the acetoxymethyl esters to liberate calcein, a very polar fluorescent dye, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time (James et all, 2002). The fluorescence from cytosolic calcein is reduced by the addition of $CoCl_2$, while the fluorescence from the mitochondrial calcein is maintained (Figure II.3.1.8).

Increasing Ca^{2+} concentration have triggered MTP-opening by ionomycin and A23187 administration (calcium ionophore) (Figure II.3.1.9). The calcium ionophore response can be blocked with cyclosporine A, a compound reported to prevent mitochondrial transition pore formation by binding cyclophilin D (Cioloca et al, 2016).

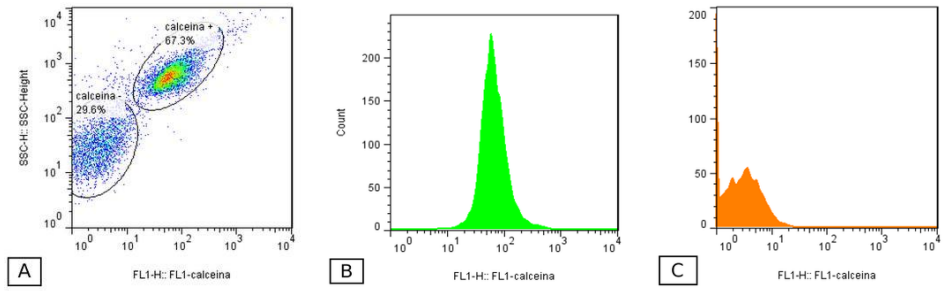

Figure II.3.1.8. *Representative FACS image for the calcein loading of normal gingival fibroblasts: A – FACS image, B and C – fluorescence histograms; FACS: Fluorescence - activated cell sorting*

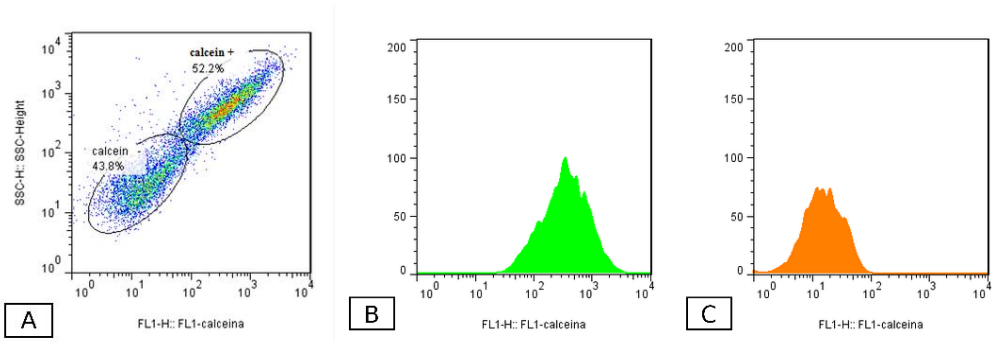

Figure II.3.1.9. *Representative FACS image for the calcein loading of normal gingival fibroblasts under ionomycin action: A – FACS image, B and C – fluorescence histograms; FACS: Fluorescence - activated cell sorting*

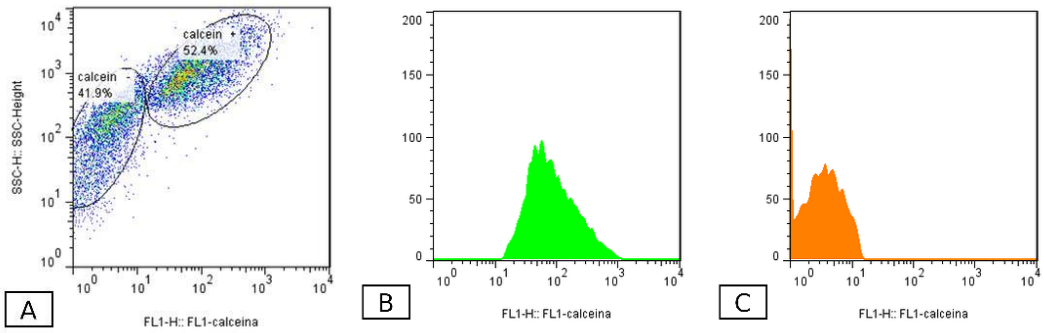

Figure II.3.1.10. *FACS image for the calcein loading of normal gingival fibroblasts under A23187 ionophore action for 24 h: A – FACS image, B and C – fluorescence histograms. FACS: Fluorescence - activated cell sorting*

Specific methods developed for measuring Ca^{2+} concentration in the cytoplasm of living cells are important in understanding the control mechanisms of calcium homeostasis (Mattson et al, 2003). At the level of the inner mitochondrial membrane there is a non - specific pore, whose opening is regulated by the level of calcium in the mitochondria, pH and membrane potential (Park et al., 1994).

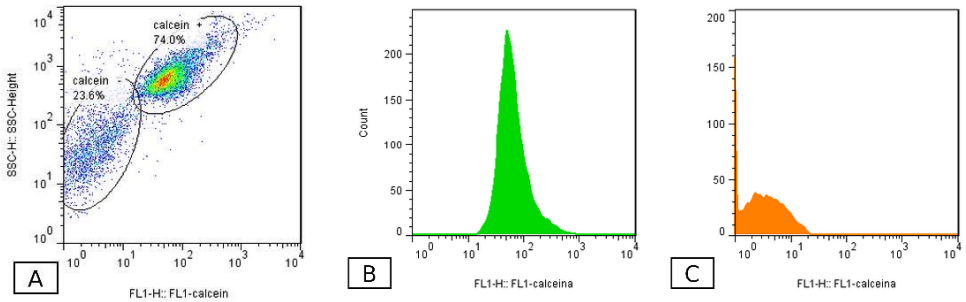

Figure II.3.1.11. *FACS collected images for the calcein loading of CsA treated gingival fibroblasts: A –FACS image, B and C – fluorescence histograms; FACS: Fluorescence - activated cell sorting; CsA: Cyclosporine A*

Magnesium ions, ATP and some antioxidants, sphingosine, carnitine and CsA

block the opening of the mitochondrial pore (Wong et al, 2012). The opening of the PTM is blocked by the CsA. This way, Ca^{2+} cannot exit the mitochondria by this route, or the rate of its efflux is very low (Silvestri et al., 2000).

The chronic treatment with CsA inhibits the transitory mitochondrial permeability, which occurs in the presence of Ca^{2+} in the mitochondria isolated from rat gingival fibroblasts. It thus becomes obvious that CsA experimentally - induced gingival overgrowth is due to the breakdown of the gingival cell homeostasis rate (Mârţu et al, 2016).

By blocking transitory mitochondrial permeability, a fundamental component of apoptosis, cellular life span is extended and further results in hyperplasic fibrosis occurring with the gingival overgrowth, induced by the chronic treatment with CsA (Diaconu et al, 2014).

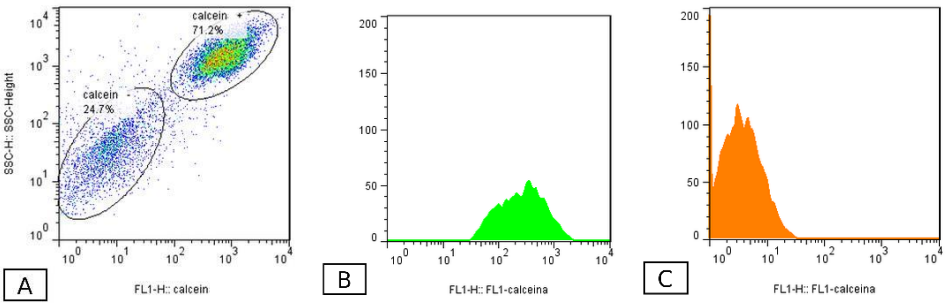

Figure II.3.1.12. *Representative FACS image for the calcein loading of CsA treated gingival fibroblasts under ionophore A23187 action: A – FACS image, B and C – fluorescence histograms; FACS: Fluorescence - activated cell sorting*

Since the dissipation of the mitochondrial membrane potential could lead to cell death, CsA might prevent this event, being demonstrated *in vitro* with gingival fibroblasts (Bernardi, 1992). Moreover, although neglected for a long time, the ability of mitochondria to function as a buffer for cytoplasmic calcium is significant, as long as they can take in significant amounts of Ca^{2+} (Park et al, 1997). However, exceeding a certain threshold, important changes in the permeability of mitochondria are triggered, which subsequently result in the release of their Ca^{2+} content, their swelling and the decoupling of oxidative phosphorylation from electron transport (Veisa et al., 2016; Wang, 2003).

The isolated mitochondrion can suffer a dramatic increase of its permeability to ions and solutions, known as "transient permeability". The transient permeability is much easier to observe after Ca^{2+}-dependent stimulation in the presence of various "inducing agents", and can be specifically inhibited by the cyclic immunosuppressive peptide, which is cyclosporine A (Wang, 2003).

II.3.1.4. DISCUSSIONS

Gingival hyperplasia has been currently associated to CsA and Nifedipine treatment. A gingival hyperplasia that is similar to the one occurring in human patients can be induced by these drugs in experimental models of rats as well (Carranza, 1996).

Whether a relationship exists between the dose and the incidence or the severity of the induced gingival overgrowth remains controversial. It was also proposed that susceptibility or resistance to drug - induced gingival overgrowth may be controlled by the existence in each individual of different proportions of fibroblast subsets, which exhibit different fibrogenic responses to the medication (Surlin et al., 2010).

So far, proof of gingival inflammation as an important factor for severity of CsA - and Nifedipine - induced gingival hyperplasia is debatable, being obvious that an optimal oral hygiene rate can minimize the severity of drug-induced gingival hyperplasia, as it eliminates the inflammatory component (Thomason et al., 1993).

Several factors, such as age, genetic predisposition, the presence of bacterial plaque and gingival inflammation, influence the occurrence of this condition. The response of patients to drugs, including immunosuppressive and antihypertensive medication, is variable (Nakib, Ashrafi, 2011). On the other hand, the awareness of this effect of the drugs on gingival tissues is minimal within the medical community (Flynn et al., 2006).

Moderate to severe forms of gingival overgrowth can damage oral hygiene and may lead to increased accumulation of microorganisms (Bosinceanu et al., 2014). Oral infections caused by these microorganisms could potentially compromise the general health of patients. (Guncu et al., 2007).

Identification and exploration of possible risk factors relating to both prevalence and severity of drug - induced gingival overgrowth would be thus very fruitful (Seymour et al, 2000; Moffitt et al, 2013). An effective management of these patients, which should minimize the possibility of complications, clearly requires the active involvement of both dental and medical professionals (Thompson et al., 2004).

Newer molecular approaches are needed to clearly establish the pathogenesis of drug - induced gingival overgrowth and to provide novel information for the design of future preventive and therapeutic strategies. Recent studies indicate salivary markers, such as interleukin-1β type cytokines, as reliable predictors of periodontal inflammation along with drugs such as Cyclosporine A, Nifedipine and Phenytoin (Luchian et al., 2016).

II.3.1.5. CONCLUSIONS

The investigated drug treatments, resulted in morphological changes and an exacerbated proliferation that enhanced with drug exposure time. Statistical analysis of normal and treated fibroblasts under the action of Ca^2+ ionophore A23187 and ionomycin showed a significant difference due to the use of ionophore A23187.

Fibroblasts previously treated with CsA have a strong dissipation of mitochondrial membrane potential under the action of this ionophore. For ionomycin, statistical analysis did not reveal critical differences for MTP opening, either in normal fibroblasts and those treated with CsA.

Mitochondrial permeability transition pore opening under Ca^{2+} overload was observed using ionophore A23187, both in normal fibroblasts and especially in those treated with CsA in culture medium. On the other hand, ionomycin, did not prove to be significant upon mitochondrial calcein load in normal or treated fibroblasts.

II.3.2. Qualitative and quantitative investigation methods of various microbiological agents and pharmacological compounds

II.3.2.1. State of the art

Nowadays oral infections can modify teeth morphology due to the acute and chronic diseases. Children being more exposed to pathogen agents, new nano-delivery system can be applied even in orthodontics. Dental carries and periodontal diseases are characterized by deterioration of the teeth and inflammation/ degradation of the periodontal tissues. If left untreated, periodontal pockets can lead to deposition of calculus, finally resulting in the loss of teeth (Pihlstrom et al., 2005; Dias el al., 2016).

Doxycycline is an antimicrobial drug used to treat extracellular and intracellular infection caused by Gram (-) and Gram (+) bacteria as well as spirochetes, *Chlamydia*, mycobacteria, mycoplasma (Riond et al., 1988; Joshi et al., 1997). This antibiotic is one of the most prescribed medicine worldwide due to its antibacterial effect on various pathogens (Cunha et al. 2000; Angelakis et al., 2015). Usual administration of doxycycline may cause side effects resulting in tissues altering, cavities in the body and blood vessels.

Nano particles antibiotic delivery carriers were designed for drug targeting to the affected site for better efficacy (Tilakaratne et al., 2014; Zegan et al., 2019).

Chitosan is a potential biomaterial for dental uses due to its special properties such as bioactivity, biocompatibility and antimicrobial (Zegan et al., 2018; De Carvalho et al., 2011; Konovalova et al., 2017; Chen et al., 2011). In the form of nanoparticles, chitosan can be used to deliver drugs to periodontal tissues in situ against microbial infections. (Samprasit et al., 2015).

The contributing papers to this direction are:

1. Zegan G, **Toma Vasilica,** Cernei ER, Anistoroaei D, Carausu EM, Moscu M. Study on Antibiotic Loaded Nanoparticles for Oral Infection Treatment. *Rev Chim*. 2019; 70(5); 1712 - 1714. (IF 2017= 1,412);
2. Mares M, Minea B, Nastasa V, Rosca I, Bostanaru AC, Marincu I, **Toma Vasilica**, Cristea VC, Murariu C, Pinteala M. *In vitro* activity of echinocandins against 562 clinical yeast isolates from a Romanian multicentre study. *Medical Mycology*, 2018; 56(4): 442 - 451. (IF 2017= 2,799);
3. Racovita S, Bunia I, Plesca I, Vasiliu S, Profire L, Foia L, **Toma Vasilica**. Release studies of cefotaxime sodium salt from coated ion exchange resin microparticles. *Farmacia*. 2017; 65(6):832 - 836. (IF = 1,507);
4. Nănescu S, Mârțu S, Ciomaga G, **Toma Vasilica**, Forna D, Foia L. Dual effects of flavonoids on dyslipidemia and periodontal disease. *Romanian Journal of Oral Rehabilitation*, 2011; 3(4):38 - 45.

II.3.2.2. MATERIALS AND METHODS

Our main goal was to investigate the potential of nanoparticles as carriers for antibiotics used in oral infections treatment. The main reagents used consisted of the common antibiotic – Doxycycline and chitosan as environmental loading agent (Figure II.3.2.1).

Chitosan nanoparticles were obtained by ionic gelation method using different concentrations of polymer dissolved in acetic acid solution.

Antibiotic - loaded chitosan nanoparticles were spontaneously formed by drop - wise addition of doxycycline to the chitosan solution before the thymidine pyrophosphate - TPP addition. Doxycycline encapsulated chitosan nanoparticles using 0.42% w/v TPP are refferred to as Doxy - CNPs4 and those prepared using 0.60% w/v TPP were referred to as Doxy - CNPs6. All nanoparticles analyses were performed by advanced characterization techniques.

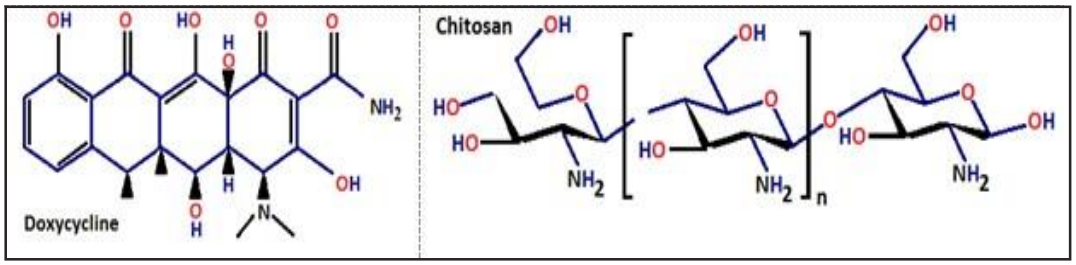

Figure II.3.2.1. *Schematic representation of doxycycline and chitosan*

II.3.2.3. RESULTS AND DISCUSSIONS

Targeting exploration of nanoparticles as carriers for antibiotics used in oral infections treatment, particle size distribution was determined measuring dynamic light scattering by particles in solution. The analysis consists of suspension of nanoparticle samples in 1 ml of pure water. The antibiotic - loaded nanoparticles Doxy - CNPs4 (fig. 2A) have an average particles diameter of 45 nm, spherical in shape with smooth edges. Similarly, the Doxy - CNPs6 particles (fig 2B) have an average diameter of 280 nm exhibiting a very narrow particle size distribution feature, spherical and with not as smooth edges than the other sample.

FTIR spectra of doxycycline, chitosan and antibiotic - loaded chitosan nanoaprticles are shown in Figure II.3.2.2. Antibacterial activity of doxycycline encapsulated chitosan nanoparticles (presented in Figure II.3.2.3) was evaluated by minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC).

Results revealed that after four hours incubation at 37°C, the antibiotic - loaded nanoparticles MIC of doxycycline was 16µg/mL for Doxy - CNPs4 and 13µg/mL for Doxy - CNPs6. For both samples, more than 90% bacteria growth inhibition was observed. MBC was 48µg/mL for Doxy - CNPs4 and 40µg/mL Doxy - CNPs6. If the incubation period was extended beyond 4 h, the nanoparticles antibacterial activity would have been higher than un-encapsulated doxycycline.

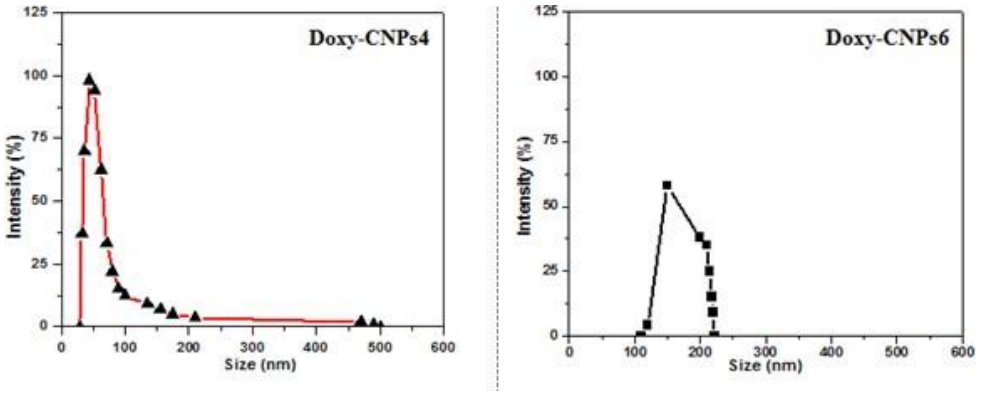

Figure II.3.2.2. *Particle size distribution for antibiotic loaded - chitosan nanoparticles: Doxy - CNPs4 and Doxy - CNPs6*

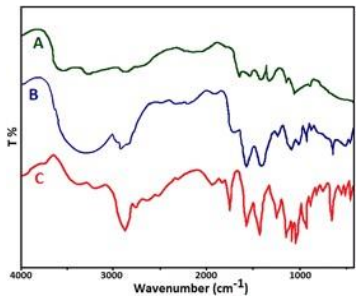

Figure II.3.2.3. *FTIR spectra of (A) Chitosan (B) Doxycycline and (C) Doxy - CNPs*

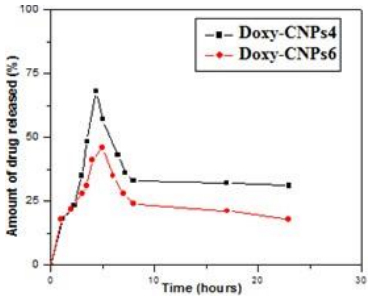

Figure II.3.2.4. *The amount of antibiotic release over a 24 - h period for Doxy - CNPs4 and Doxy - CNPs6*

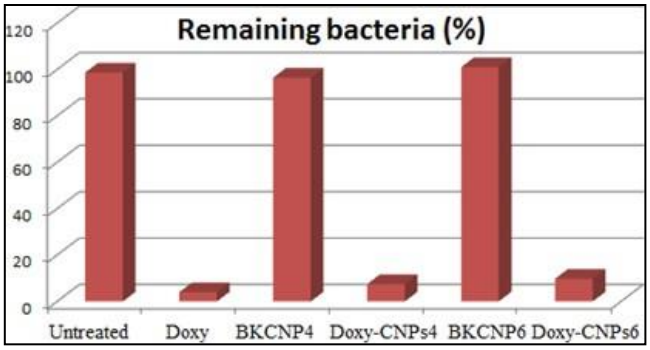

Figure II.3.2.3. *Inhibitory effects of antibiotic - encapsulated chitosan nanoparticles on bacterial growth, in terms of percentage of remaining bacteria after 4 h of treatment*

The main driving force in the advanced investigations and applications of chitosan comes from its naturally abundant, satisfactorily biocompatible, biodegradable and nontoxic. Many pharmaceutical applications have been focused on chitosan since its structure is cellulose-like, and the free amino groups on this polymeric chain contribute the reactive and polycationic nature that exhibit complexation properties. The potential applications of chitosan as an excipient in oral formulation and other delivery systems are

reported, and that is why we were interested in nanoparticles formulations for oral bacterial infection treatment. Doxycycline (α-6-deoxy-5-oxytetracyclin), a well-known broad-spectrum antibiotic, exhibits bacteriostatic activity, inhibiting the bacterial protein synthesis due to the disruption of transfer RNA and messenger RNA at the ribosomal sites.

II.3.2.4. CONCLUSIONS

- We presented a nanoparticulate formulation of doxycycline - encapsulated chitosan nanoparticles that display preliminary promise for possible eventual use in drug delivery and improved efficacy in the treatment of oral bacterial infections.
- The results suggest that this formulation has the potential to control oral infections in a sustained manner which can be a strategy for biofilm - associated infection treatment.

Section III

FUTURE EVOLUTION AND DEVELOPMENT PLANS UPON CAREER AND RESEARCH ACTIVITY

During my entire years of activity, both on the academic field and dental practice, I tried to manage a proper harmonius link between the clinical research and didactic activity, a permanent improvement in Pedodontics teaching and scientific progress being the main goal of my previous activity. In my opinion, the purpose of medical education is to develop capable primary clinicians and to contribute to world health through medical research. Medical research plays an important role in basic clinical practice as it provides us with evidence and rationale for our actions. The educational goals of my future career will target provision students with appropriate objectives and motives, searching for what is best in order to pass the new information on to dental students.

Considering that in present, modern medical professionalism serves as a standard of care and plays an important role in the improvement of the patient's treatment, I am pretty much sure that such standards should be further refined by developing clinical knowledge, communication skills, and teamwork skills.

III.1. FUTURE DIRECTIONS IN RESEARCH ACTIVITY

On short, medium and long term, my research activiy will be guided toward:

- Carry on and settling the research of the child on the level of oral cavity status, supporting the scientific research reports;
- Dissemination of results in ISI / indexed international databasis journals;
- Continuing and expanding the collaboration with the preclinical disciplines such as Immunology, Biochemistry, Medical Genetics, Pharmacology and other dental disciplines (Prevention, Caryology, Endodontics, Periodontology, Orthodontics, Prosthetics), addressing new proposals that could provide real funding through joint research projects;
- Attracting funds to improve the means of research at the Pedodontics discipline;
- Expanding collaboration with other research laboratories outside the faculty;
- Organizing and participating in national and international scientific events;
- Expanding pediatric research through participation in national grants;
- Developing applications for international research programs and grants;
- Organization of student scientific events, for the purpose of their involvement and inclusion in the research activity and participation in scientific events, as well;

The human body is very complex and continuously challenging, therefore only interdisciplinary research can integrate data, methodologies, perspectives, and concepts from various fields to characterize its homeostasis.

The major path of my future academic research will concern directions and areas that are already in progress, involving activities that made the fundamental of my last two years preocupations:

- **Prevention of musculoskeletal disorders in dentistry,** because the appropriate timing of orthodontic intervention has been an intriguing debate among specialists due to lack of solid scientific evidence.

The effectiveness of the intervention depends largely on the type of malocclusion. Most often, the main issue is the benefit obtained by treating developing malocclusions in the early mixed dentition stage when compared with treatment started in the late mixed dentition or in the permanent dentition, when malocclusion has already been established.

One reason for the controversy is that the implied toll of an early treatment time is a two-phase protocol. Phase 1 usually takes 6 up to 12 months of active treatment with the intent to augment dento-skeletal relationships. Phase 2 is the "finishing" step after the eruption of the permanent teeth. In this situation, we should carefully analize the risk/benefit of an early intervention so as to justify the potential added cost of two-phase treatment; Preventive orthodontics are procedures aimed at promoting the development of a normal occlusion and support the prevention of malocclusion from developing, whereas interceptive orthodontics encourages the restoration of a normal occlusion once a malocclusion has started to develop. Genetic and environmental factors can contribute to the progress of such a pathology and can span several years, thus making it difficult to determine specific causative factors.

- **Evaluation of the link between infectious diseases such as Hepatitis C Infection and Periodontal Disease** - Hepatitis C virus (HCV) infections could have an important impact on the oral health status of patients, favoring conditions such as periodontal disease and oral cancer. The changes caused by the infection in the subjects' immune system, diet, and lifestyle can facilitate the development of oral conditions such as periodontal disease. Important changes also occur in the composition of the infected patients' saliva and gingival fluid. HCV-infected patients need to be carefully monitored in terms of oral health since the infection with the virus can result in oral complications. I also intend to pursue upon the research on the presented directions as well as to extend the area of interest to other innovative diagnostic systems in the context of the fulminant advances in the medicine field.

- **Dental eruption disorders in mentally challenged patients -** By its localization, the impacted central incisor represents a serious aesthetic problem for parents. As they represent the most prominent teeth in a patient's smile and are usually the most visible during speech, their eruption and position can have a major impact on dental and facial aesthetics in patients. Failure of eruption can be considered as esthetically unappealing and may influence self-esteem and confidence in developing social relationships with others.

The maxillary central incisor is the third ranked teeth regarding impactions, after

third molars and maxillary canines. Males are affected more frequently than females and this anomaly is more common when there are other inherited dental anomalies such as: enamel hypoplasia, supranumerary teeth and other ectopic anomalies. The occurrence of impacted maxillary central incisors can be associated with many hereditary, developmental and environmental factors, the importance of these factors being not yet clarified. Most of the impacted central incisors are diagnosed at 7–8 years of age, and thus treatment should be initiated if the orthodontist is confident that the patient will be able to handle and understand in a proper manner what the treatment encompasses. There is no definitive evidence to state that the younger the patient, the more adequately the tooth will erupt, but there are significant psychosocial advantages from early alignment. Several approaches have been advocated as treatment, but they all share the common point that, it is best to avoid a prolonged dental eruption, for a more advantageous outcome.

These studies follow the actual trend of experimental and clinical researches in pedodontics, needing a tight and close cooperation between experienced dental and general practice specialists in the field.

III.2. FUTURE DIRECTIONS IN TEACHING ACTIVITY

"Education is the acquisition of the art of using knowledge"
(Alfred North Whitehead**)**

Linking research to educational and medical activities requires either implementing research results in medical practice and educational process, coordination of research topics within student scientific meetings, the progress in the teaching stages according to the acquired skills and the existing opportunities, while increasing the scientific reputation of the department / faculty / university, as well.

A good training in pedodontics entails the acquiring of the skill of rational diagnosis and cure, together with the ability to transmit the information, and that is why my future teaching directions include:

- Systematization of a teaching protocol for courses and clinical practice,
- The individualization of the didactic protocol - adaptation of teaching level, professional-scientific language used and terminology, according to the level of medical culture (both in dentistry and general medicine), year of study;
- The adaptation of the didactic material - the harmonious utilization of the existing technical and material resources within the discipline for the support of the lectures and the practical training, to achieve an effective management of teaching time by using a course pattern to support understanding and preservation of the new information;
- Supporting educational material with clinical examples, complex eloquent case studies, practical demonstrations, multi-media presentations;

- Improving the didactic practice approaches by applying an active-participatory strategy with the student's involvement in the teaching-learning process, solving hypothetical clinical situations;
- Keep up-to-date the curriculum content, clinical traineeship and provision of the information, in line with pedodontics novelties and technology capabilities;
- Stimulating students to develop behavioral and therapeutic behavioral considerations in Pedodontics;
- Gathering information and data collection, so that, together with the members of the dental team, to issue a practical guide, as well as a book volume in the specialty of Pediatric periodontiology for the French line studies;
- Active participation in national and international scientific events;
- Diversification of students' assessment techniques based on student performance and skills, periodic evaluation of the student's tasks in order to become familiar with the methods of final examination and evaluation;
- Collaboration with other national centers to facilitate student mobility;
- Creating a strong teamwork within the Pedodontics discipline for interested and skilled students, in which to be able to carry out short-term research programs.

The student-centered teaching method will be continued with the future involvement of the student in practical virtual cases that needs the application of specific knowledge, in order to interconnect the practical and theoretical data from lectures and applied activities. The postgraduate programs with master students and resident doctors in orthodontics, endodontics, surgery, prosthetics will continue during the pedodontics module as well, to stimulate the young doctors to discover that the challenging aspects of the research activity are based on creativity and on solid knowledge.

Section IV

REFERENCES

1. Abdel-Salam E, Abdel-Meguid I, Korraa S. Assessment of immune function in Down syndrome patients. *Egypt J Med Hum Genet*.2013;14:307---10.16.
2. Aburawi E, Sould A. Lymphocyte respiration in children with trisomy 21. *BMC Pediatr* 2012;12:193–8.
3. Açıkgöz G, Cengiz MI, Keskiner I, Açıkgöz S, Can M, Açıkgöz A. Correlation of hepatitis C antibody levels in gingival crevicular fluid and saliva of hepatitis C seropositive hemodialysis patients. *International Journal of Dentistry, 2009*.
4. Adumitroaie A, Foia L, Anistoroaei D, Cioloca D, Maftei G, Bogdan M, Vlad C, Toma V. Review of the correlation between specific biomarkers in leukemia and periodontal disease in children. *Romanian Journal of Medical and Dental Education*, 2019; 8(3): 23-27.
5. Akalin FA, Toklu E, Renda N. Analysis of superoxide dismutase activity levels in gingiva and gingival crevicular fluid in patients with chronic periodontitis and periodontally healthy controls. *J Clin Periodontol* 2005; 32:238–243.
6. Al-Khabbaz a Khalaf F Areej K.. Al-Shammari a Abdulaziz Hasan,Majedah Abdul-Rasoul : Periodontal Health of Children with Type 1 Diabetes Mellitus in Kuwait: A Case-Control Study. *Medical Principles and Practice*, 2013; 22:144–149 ; DOI: 10.1159/000342624.
7. American Cancer Society. *Global Cancer Facts & Figure*, 2nd ed., Atlanta, American Cancer Society, 2011. Available from: http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-027766.pdf
8. American Diabetes Association. (2006). Standards of medical care in diabetes, *Diabetes Care* 29 (Suppl. 1): S4–S42.
9. Anders PL, Davis EL. Oral health of patients with intellectual disabilities: a systematic review. Spec Care Dentist. 2010; 30: 110–117.
10. Angelakis, E., Armstrong, N., Nappez, C., et al. *Infect* 2015; 71, p. 511.
11. Aren G, Sepet E, Ozdemir D, Dinccag N, Guvener B, Firatli E: Periodontal health, salivary status, and metabolic control in children with type 1 diabetes mellitus. *J Periodontol* 2003; 74: 1789–1795.
12. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol.* 1999; 4: 1–6.
13. Armitage, GC. Development of a classification system for periodontal diseases and conditions. *Northwest Dent.* 2000; 79(6):31-5.
14. Balan A, Balan G, Toma V. Religion, subjectivity and oral health ethical dilemmas

in pediatric dentistry. *European Journal of Science and Theology*, 2014; 10(1): 155-165.
15. Balan A, Maxim A, Pasareanu M, Toma V, Balcos C, Barlean L. Socio-epidemiological aspects of odonto-periodontal pathology in the young adult between decades and actuality. *International Journal of Medical Dentistry* 2012; 16(3):167-171.
16. Baliga AM, Brave VR, Vyas HA. Oral mucosal lesions in patients with acute leukemias and related disorders due to cytotoxic therapy. *J Indian Soc Pedod Prev Dent.* 1995; 13(1):25-9.
17. Barros SP, Williams R, Offenbacher S, Morelli T. Gingival crevicular fluid as a source of biomarkers for periodontitis. *Periodontology 2000*, 2016; 70(1): 53-64.
18. Bastos JV, Queiroz-Junior CM, Caliari MV, Francischi JN, Pacheco CM, Maltos KL. Peripheral kappa opioid receptors activation reduces alveolar bone loss in rats by modulating interleukin-6 and -10. *Arch Oral Biol.* 2011; 56(6):540-548.
19. Becerik S, Özçaka Ö, Nalbantsoy A, Atilla G, Celec P, Behuliak M, Emingil G. Effects of menstrual cycle on periodontal health and gingival crevicular fluid markers. *Journal of Periodontology*, 2010; 81(5), 673-681.
20. Bernardi P, Modulation of the mitochondrial cyclosporine A sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J. Biol. Chem.*, 1992; 267:8834-8839.
21. Berry JD, Dyer A, Cai X, Garside DB, Ning H, Thomas A, Lloyd-Jones DM. Lifetime risks of cardiovascular disease. *New England Journal of Medicine*, 2012; 366(4): 321-329.
22. Bloemers BLP, Bont L, De Weger RA, Otto SA, Borghans JA, Tesselaar K. Decreased thymic output accounts for decreased naive T cell numbers in children with Down syndrome. *J Immunol* 2011; 186:000.
23. Bonito AJ, Lux L, Lohr KN. Impact of local adjuncts to scaling and root planing in periodontal disease therapy: A systemic review. *J Periodontol*. 2005; 76: 1227–1236.
24. Borgnakke WS, Ylöstalo PV, Taylor GW, Genco RJ. Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence. *J Clin Periodontol.* 2013; 40(suppl 14):S135-S152.
25. Bosinceanu DN, Sandu IG, Bosinceanu DG, Forna NC. Study of Candida albicans colonies on a new polymer used to create complete dentures. I. Rev Chim (Bucharest), 2014, 65(4):466–469.
26. Bours M, Swennen E, Di Virgilio F, Cronstein B, Dagnelie P. Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther* 2006; 112:358.
27. Boyd LD, Giblin L, Chadbourne D. Bidirectional relationship between diabetes mellitus and periodontal disease: state of the evidence. *Can J Dent Hygiene.* 2012; 46(2):93-102.
28. Brill N, Krasse BO. The passage of tissue fluid into the clinically healthy gingival pocket. *Acta Odontologica Scandinavica*, 1958. 16(3), 233-245.

29. Buduneli N, Kinane DF. Hostderived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *Journal of Clinical Periodontology*, 2011; 38, 85-105.
30. Burke VP, Startzell JM, The Leukemias. *Oral Maxillofac Surg Clin North Am*. 2008 Nov; 20(4): 597-608.
31. Campo E, Swerdlow SH, Harris NL, Harris N, Pileri S, Stein H, Jaffe E. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood* 2011; 117:5019-32.
32. Caraiane A, Toma V, Raftu G, Debita M, Dimofte AR, Iordache C. Aesthetic Rehabilitation of the Teeth using Single Fixed Prostheses. *Rev Chim*. 2019; 70(2): 714-717.
33. Carey AJ, Tan CK, Ulett GC. Infection-induced IL-10 and JAK-STAT: A review of the molecular circuitry controlling immune hyperactivity in response to pathogenic microbes. JAKSTAT. 2012; 1:159–67.
34. Carollo-Bittel B, Persson RE, Persson GR, Lang NP. Supportive periodontal therapy for high- and low-risk patients. *J Investig Clin Dent.* 2011; 2(2):110-6.
35. Carranza FA Jr. Gingival enlargement. In: Carranza FA, Newman MG (eds). *Clinical periodontology. 8th edition, W.B. Saunders & Co., Philadelphia*, 1996; 235–238.
36. Carroll WL, Bhojwani D, Min DJ, Raetz E, Relling M, Davies S, Downing JR, Willman CL, Reed JC. Pediatric acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program* 2003; 102-31.
37. Casamassimo PS, Flaitz CM, Hammersmith K, Sangvai S, Kumar A. Recognizing the relationship between disorders in the oral cavity and systemic disease. *Pediatr Clin N Am.* 2018; 65: 1007-1032.
38. Casamassimo PS, Thikkurissy S, Edelstein B, Maiorini E. Beyond the dmft: the human and economic costs of early childhood caries. *J Am Dent Assoc*. 2009; 140:650-7.
39. Cavalcante LB, Tanaka MH, Pires JR, Apponi LH, Aparecida Giro EM, Valentini SR, Palomari Spolidório DM, Capela MV, Rossa C Jr, Scarel-Caminaga RM. Expression of the interleukin-10 signaling pathway genes in individuals with Down syndrome and periodontitis. *J Periodontol.* 2012; 83(7):926-35.
40. Centers for Disease Control and Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. *Atlanta: CDC.*
41. Cergizan D, Branasco T, Brujbu IC, Toma V, Iordache C. Restoration the Dent-stomato-facial aesthetic balance, a target of dental therapy, regardless the patient's age. *Rev Chim*. 2017; 68(10): 2358-2362.
42. Chapple, I. L., Matthews, J. B. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontology 2000*. 2007; 43, 160–232.
43. Chen M.I., Liao Z., Sung H. Chitosan, in Chitosan for Biomaterials I; *Springer: Berlin/Heidelberg, Germany*, 2011; p. 185.
44. Chen, X, WU, G, Feng Z, Dong Y, Zhou W, Li B, Bai S, Zhao Y, Crit Rev. *Biotechnol.* 2016; 36, p. 760.

45. Cheng RH, Leung WK, Corbet EF. Non-surgical periodontal therapy with adjunctive chlorhexidine use in adults with Down syndrome: a prospective case series. *J Periodontol*. 2008; 79: 379–385.
46. Cichon P, Crawford L, Grimm WD. Early-onset periodontitis associated with Down's syndrome- a clinical interventional study. *Ann Periodontol.* 1998; 3: 370–380.
47. Cinek O. Epidemiology of childhood type 1 diabetes mellitus: lessons from central and Eastern European data. *Hormone Research in Paediatrics* 2011; vol. 76, no. 1, pp. 52–56.
48. Cioloca DP, Foia L, Holban C, Trandafirescu M, Poroch V, Maxim D, Jipu R, Costuleanu M, Toma V. Systemic diabetic context-induced biochemical periodontal alterations in children. *Rev Chim.* 2016; 67(12): 2409-2412.
49. Cioloca DP, Foia L, Holban C, Trandafirescu M, Poroch V, Maxim D, Jipu R, Costuleanu M, Toma Vasilica. Systemic diabetic context-induced biochemical periodontal alterations in children. *Rev Chim*. 2016; 67(12): 2409-2412.
50. Cioloca DP, Ursarescu I, Martu A, Toma V, Surdu A, Botnariu G, Bogdan M, Caruntu ID. Systemic and periodontal inflammatory burden in children and teenagers with diabetes and clinical correlations. *The Medical-Surgical Journal*, 2015; 119(3): 896-902.
51. Cioloca DP, Ursarescu I, Martu A, Toma V, Surdu A, Botnariu G, Bogdan M, Caruntu ID. Systemic and periodontal inflammatory burden in children and teenagers with diabetes and clinical correlations. *The Medical-Surgical Journal*, 2015; 119(3): 896-902.
52. Clarke CM, Edwards JH, and Smallpeice V, The Lancet, 185 (1961) 1028-1030.
53. Cohen MM, Winer RA, Schwartz S, Shklar G, Oral aspects of mongolism. I. Periodontal disease in mongolism. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics 1961; 14: 92-107.
54. Costa, FO., Cota, LOM., Costa, JE.& Pordeus, IA. Periodontal disease progression among young subjects with no preventive dental care: A 52-month follow-up study, *J Periodontol* 2007; 78(2): 198-203.
55. Cunha B.A., Domenico P., Cunha C.B. Pharmacodynamics of doxycycline. *Clin. Microbiol. Infect*., 6, no. 5, 2000, p. 270-273.
56. Curtis AB. Childhood leukemias: Initial oral manifestations. *J Am Dent Assoc*. 2001; 83:159-64.
57. Curtis MA, Griffiths GS, Price SJ, Coulthurst S K, Johnson, NW. The total protein concentration of gingival crevicular fluid: Variation with sampling time and gingival inflammation. *Journal of Clinical Periodontology*, 1988. 15(10), 628-632.
58. D.U. Ahn, K.C. Nam. Effects of ascorbic acid and antioxidants on colour, lipid oxidation and volatiles of irradiated ground beef. *Radiation Physics and Chemistry* 2004; 71: 149-154.
59. Dakovic D, Pavlovic MD: Periodontal disease in children and adolescents with type 1 diabetes in Serbia. *J Periodontol* 2008; 79:987–992.

60. De Carvalho M., Stamford T., Pereira E., Dos Santos P., Sampaio F. Chitosan as an oral antimicrobial agent. *Formatex* 2011; 13, p. 2012.
61. De Pomereau, V., Dargent-Pare, C., Robert, JJ. & Brion, M. Periodontal status in insulin dependent diabetic adolescents, *J Clin Periodontol*. 1992; 19(9): 628-32.
62. Delima AJ, Van Dyke TE. (2003). Origin and function of the cellular components in gingival crevice fluid. *Periodontology 2000*, 31(1), 55-76.
63. Demmer RT, Holtfreter B, Desvarieux M, et al. The influence of type 1 and type 2 diabetes on periodontal disease progression: prospective results from the Study of Health in Pomerania (SHIP). *Diabetes Care* 2012; 35(10):2036-2042.
64. Detert J, Pischon N, Burmester GR, Buttgereit F. The association between rheumatoid arthritis and periodontal disease. *Arthritis Research & Therapy*, 2010; 12(5): 218.
65. Dias R.J., Havaldar V.D., Ghorpade V.S., Mali K.K., Gaikwad V.K., Kumbhar D.M. Development and Evaluation of In-Situ Gel Containing Ornidazole Loaded Microspheres for Treatment of Periodontitis. *J App Pharm Sci*, 2016; 6 (10): 200-209.
66. Dinarello CA. Pro-inflammatory cytokines. *Chest 2000*;118:503–508, Down Syndrome-Fact Sheet. *National Down Syndrome Society (NDSS).* 2015;1.
67. Dow RS, A preliminary study of periodontoclasia in Mongolian children at Polk State Scholl, *Am J Ment Defic*.1951; Apr;55(4):535-8.
68. Down JLH, Clinical Lecture Reports. London Hospital, 1866, 3:259–262.
69. Du S., Jia Y., Tang H., Sun Y., Wu W., Sun L., Du J., Geng B., Tang C., Tin H. Immune regulation of hydrogen sulfide in children with acute lymphoblastic leukemia. *Chin Med J (Engl),* 2014; 127:3695-3699.
70. Duell BL, Tan CK, Carey AJ, Wu F, Cripps AW, Ulett GC. Recent insights into microbial triggers of interleukin-10 production in the host and the impact on infectious disease pathogenesis. FEMS Immunol Med Microbiol. 2012; 64:295–313.
71. Eden T, Aetiology of childhood leukaemia. *Cancer Treat Rev.* 2010; 36:286-297.
72. Egelberg J, Attström R. (1973). Comparison between orifice and intracrevicular methods of sampling gingival fluid. *Journal of Periodontal Research*, 1973. 8(6): 384-388.
73. Egelberg J. Permeability of the dento-gingival blood vessels: I. Application of the vascular labelling method and gingival fluid measurements. *Journal of Periodontal Research*, 1966. 1(3), 180-191.
74. *Eisen D, Lynch DP*. Oral manifestations of systemic diseases. In: *Eisen D, Lynch DP* (eds). The mouth. Diagnosis and treatment. *St Louis (MO): Mosby*, 1998; 212-236.
75. Ellis, S. D., Tucci, M. A., Serio, F. G., Johnson, R. B. Factors for progression of periodontal diseases. *J. Oral Pathol. Med.* 1998; 27, 101–105.
76. Embery G, Waddington R. (1994). Gingival crevicular fluid: biomarkers of periodontal tissue activity. *Advances in Dental Research*, 8(2), 329-336.

77. Engebretson, S., Chertog, R., Nichols, A., Hey-Hadavi, J., Celenti, R. & Grbic, J. Plasma levels of tumour necrosis factor-alpha in patients with chronic periodontitis and type 2 diabetes. *J Clin Periodontol*. 2007; 34(1):18-24.
78. Esen Ç, Alkan BA, Kırnap M, Akgül Ö, Işıkoğlu S, Erel Ö. The effects of chronic periodontitis and rheumatoid arthritis on serum and gingival crevicular fluid total antioxidant/oxidant status and oxidative stress index. *Journal of Periodontology*, 2012; 83(6): 773-779.
79. Esquirol E, Baillière JB (and sons) (eds.), Des maladies mentales considérées sous les rapports médical, hygiénique et médico-légal, Vol. 1, J.B.Bailliere, Paris, 1838, 236.
80. Flynn JC, Henderson JS, Johnson RB. Synergism between nifedipine and cyclosporine A on the incorporation of [35S] sulfate into human gingival fibroblast cultures in vitro. *J Periodontal Res,* 2006; 41(4):316–321.
81. Foia L, Toma V**,** *Patologia parodontala in diabetul juvenil*. Ed "Gr. T. Popa, Iaşi, 2008.
82. Foia L, Toma V, Surlin P. Diabetes mellitus impact on periodontal status in children and adolescents. Chp. 8: p. 179-197 in Editor J. Manakil; Periodontal disease – a clinician's guide. *InTech Ed.* 2012, ISBN 978-953-307-818-2.
83. Foia L, Toma V, Ungureanu D, Aanei C, Costuleanu, M. Binomial relationship periodontal disease-diabetes mellitus: ethiologic and risk factors. *The Medical-Surgical Journal*, 2007; 111(3):748-753.
84. Foia L, Toma V, Ungureanu D, Zlei M, Indrei A, Forna D, Filip F, Nanescu S, Evaluation of the oral injuries experimental induced diabetes mellitus by analysis of some gingival fluid markers. *The Medical-Surgical Journal*, 2008; 112(4):1066-1071.
85. Foia L, Toma Vasilica: Patologia parodontala in diabetul juvenil. *Ed "Gr. T. Popa, Iaşi,* 2008.
86. Foia L, Ungureanu D, Toma V, Zlei M, Indrei A, Haba D, Branisteanu D. Analysis of oral expression of the diabetes-periodontal disease binomial relationship in a juvenile population. *Romanian Review of Laboratory Medicine*, 2008; 13(4): 39-48.
87. Freedman L, Nunn J, ThomasW, Claffey N, Kelly A. Preventive strategies to improve periodontal health in people with Down syndrome. *J Disabil Oral Health.* 2011;12:59–67.
88. Frydman A, Nowzari H. Down Syndrome-Associated Periodontitis: A Critical Review of the Literature. *Compendium* 2012; 33: 356–361.
89. Gaikwad S, Jadhav V, Gurav A, Shete AR, Dearda HM. Screening for diabetes mellitus using gingival crevicular blood with the help of a self-monitoring device. *J Periodontal Implant Sci.* 2013; 43(1):37-40.
90. Gardiner KJ. Pharmacological approaches to improving cognitive function in Down syndrome: current status and considerations. *Drug Des Dev Ther.* 2014; 9:103-25.26.
91. Gheorghe DN, Camen A, Foia L, Solomon S, Toma V, Mateescu OG, Surdu A, Rogoveanu I, Surlin P. Histologic and immunohistochemical assessment of gingival

tissue's changes induced by periodontal disease in association with chronic hepatitis C. *The Medical-Surgical Journal*, 2018; 112(4): 789-797.
92. Gheorghe DN, Foia L, Toma V, Surdu A, Herascu E, Popescu DM, Surlin P, Vere CC, Rogoveanu I. Hepatitis C Infection and Periodontal Disease: Is there a Common Immunological Link? *Journal of Immunology Research*, 2018. Article Number: 8720101, DOI: 10.1155/2018/8720101.
93. Giannopoulou C, Kamma JJ, Mombelli A. Effect of inflammation, smoking and stress on gingival crevicular fluid cytokine level. *Journal of Clinical Periodontology*, 2003. 30(2), 145-153.
94. Gokul K. Estimation of the level of tumor necrosis factor-α in gingival crevicular fluid and serum in periodontal health and disease: a biochemical study. *Indian Journal of Dental Research*, 2012; 23(3), 348.
95. Golovcencu L, Zegan G, Saveanu IC, Anistoroaei D, Toma Vasilica. Interdisciplinary orthodontics for improved dental esthetics in the frontal area; A review. *Romanian Journal of Medical and Dental Education*, 2019; 8(2):47-51.
96. Gomes FIF, Aragão MGB, Barbosa FCB, Bezerra MM, Pinto VD, Chaves HV. Inflammatory cytokines interleukin-1β and tumour necrosis factor-α-novel biomarkers for the detection of periodontal diseases: a literature review. *Journal of Oral & Maxillofacial Research*, 2016; 7(2).
97. Goodson JM. (2003). Gingival crevice fluid flow. *Periodontology 2000*, 31(1), 43-54.
98. Goriuc A, Foia LG, Minea B, Luchian AI, Surdu AE, Toma V, Costuleanu M, Martu I. Drug-induced gingival hyperplasia – experimental model. *Rom J Morphol Embryol*. 2017; 58(4): 1371-1376.
99. Goriuc A, Minea B, Foia L, Costuleanu M, Jipu R, Toma V, Vlad C, Luchian I, Martu I. The role of mitochondrial calcium overload in cyclosporine A-induced gingival hyperplasia. *Romanian Journal of Oral Rehabilitation*, 2017; 9(3): 55-61.
100. Graves DT, Cochran D. The contribution of interleukin1 and tumor necrosis factor to periodontal tissue destruction. *Journal of Periodontology*, 2003; 74(3), 391-401.
101. Griffiths GS. (2003). Formation, collection and significance of gingival crevice fluid. *Periodontology 2000*, *31*(1), 32-42.
102. Griffiths GS. Formation, collection and significance of gingival crevice fluid. *Periodontol 2000.* 2003; 31:32-42.
103. Grover HS, Luthra S, Molecular mechanisms involved in the bidirectional relationship between diabetes mellitus and periodontal disease. *Journal of Indian Society of Periodontology,* 2013; vol. 17, no. 3, pp. 292–301.
104. Güncü GN, Cağlayan F, Dinçel A, Bozkurt A, Ozmen S, Karabulut E. Clinical and pharmacological variables as a risk factor for nifedipine-induced gingival overgrowth. *Aust Dent J*, 2007; 52(4):295–299.
105. Hamertone JL, Briggs SM, Giannelli F, Carter CO. Chromosome studies in detection of parents with high risk of second child with Down's syndrome. *Lancet.* 1961; 2: 788–791.

106. Hanes Philip J, Krishna R, Characteristics of inflammation common to both diabetes and periodontitis: are predictive diagnosis and targeted preventive measures possible? *EPMA Journal* 2010; 1:101–116.
107. Hanookai D, Nowzari H, Contreras A, Morrison JL, Slots J. Herpes viruses and periodontopathic bacteria in Trisomy 21 periodontitis. *J Periodontol.* 2000; 71: 376–384.
108. Hara K, Löe H. Carbohydrate components of the gingival exudate. *Journal of periodontal research*, 1969. 4(3), 202-207.
109. Haskó G, Cronstein B. Regulation of inflammation by adenosine. *Front Immunol* 2013; 8:85.
110. Hasko G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and imune diseases. *Nat Rev* 2008; 7:759–70.
111. Hedge AM, Joshi S, Rai K, Shetty S. Evaluation of oral hygiene status, salivary characteristics and dental caries experience in acute lymphoblastic leukemia (ALL) children. *J Clin Pediatr Dent.* 2011; 35:319-23.
112. Hernandez D, Fisher E. Down syndrome genetics: unravelling a multifactorial disorder. *Hum Mol Genet* 1996; 5:1411–6.
113. Hönig J, Rordorf Adam C, Siegmund C, Wiedemann W, Erard F. Increased interleukin1 beta (IL-1β) concentration in gingival tissue from periodontitis patients. *Journal of Periodontal Research*, 1989; 24(6), 362-367.
114. Hou GL, Huang JS, Tsai CC. Analysis of oral manifestations of leukemia: a restrospective study. *Oral Dis.* 1997; 3(1):31-8.
115. Hutchins AP, Diez D, Miranda-Saavedra D. The IL-10/STAT3-mediated anti-inflammatory response: Recent developments and future challenges. Brief Funct Genomics. 2013; 12:489–477.
116. Iacopino, AM. Periodontitis and diabetes interrelationships: role of inflammation. *Ann Periodontol*. 2001; 6(1): 125-137.
117. Jacob F, Perez Novo C, Bachert C, Van Crombrugge K. Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses. *Purinergic Signal* 2013; 84:154–60.
118. James A. M., Murphy M. P. How mitochondrial damage affects cell function. *J. Biomed.Sci.* 2002; 9: 475–487.
119. Javed F, Ahmed A. Proinflammatory cytokines in the saliva, gingival crevicular fluid and serum of diabetic patients with periodontal disease. *J Res Pract Dent* 2013:1-10.
120. Javed F, AlAskar M, AlHezaimi K. Cytokine profile in the gingival crevicular fluid of periodontitis patients with and without type 2 diabetes: a literature review. *Journal of Periodontology*, 2012; 83(2), 156-161.
121. Javed F., Utreja A., Bello Correa FO., Al-Askar M., Hudieb M. Oral health status in children with acute lymphoblastic leukemia. *Crit Rev Oncol Hematol* 2012; 83:303-309.
122. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics. *CA Cancer J Clin* 2007; 57(1):43-66.

123. Jhonson N, Young MA. Periodontal disease in mongols, *Journal of Periodontology* 1963; 34: 41-47.
124. Jones CG. Chlorhexidine: Is it still the gold standard? *Periodontol 2000.* 1997; 15: 55–62.
125. Joshi N., Miller D.Q. Doxycycline revisited. *Arch Intern Med.*, 1997; 157(13):1421-8.
126. Karvonen M., M. Viik-Kajander, E. Moltchanova, I. Libman, R. LaPorte, and J. Tuomilehto. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. *Diabetes Care*, 2000; vol. 23, no. 10, pp. 1516–1526.
127. Kaste SC, Hopkins KP, Jones D, Crom D, Greenwald CA, Santana VM. Dental abnormalities in children treated for acute lymphoblastic leukemia. *Leukemia.* 1997; 11:792-796.
128. Katz J, Peretz B. Trismus in a 6 year-old child: a manifestation of leukemia? *J Clin Pediatr Dent*. 2002; 26(4): 337-9.
129. Kawashima K, Fujii T. The lymphocytic cholinergic systemand its contribution to the regulation of immune activity. *Life Sci* 2003; 74:675–96.
130. Kerr JFR, Wyllie AH, Currie AR, Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics, *Br. J. Cancer.*, 1972, 26(4):239-257.
131. Khalaf H., Lonn J., Bengtsson T. Cytokines and chemokines are differentially expressed in patients with periodontitis: possible role for TGF-β1 as marker for disease progression. *Cytokine,* 2014; 67:29-35.
132. Khurshid Z, Mali M, Naseem M, Najeeb S, Zafar M. (2017). Human gingival crevicular fluids (GCF) proteomics: an overview. *Dentistry Journal*, 5(1), 12.
133. Kolli G, Chaitra N, Ranjan V, Nateshkumar DK. Diagnosis of a case of relapse of acute lymphoblastic leukemia based on oral manifestation of leukemic gingival enlargement and acute necrotizing gingivitis: A case report. *J Indian Acad Oral Med Radiol.* 2014; 26:347-50.
134. Konovalova M.V., Markov P.A., Durnev E.A., Kurek D.V., Popov S.V., Varlamov V.P., Preparation and biocompatibility evaluation of pectin and chitosan cryogels for biomedical application. *J. Biomed. Mater. Res.* 2017; 105:547–556.
135. Koregol A C, More S P, Nainegali S, Kalburgi N, Verma, S. Analysis of inorganic ions in gingival crevicular fluid as indicators of periodontal disease activity: A clinico-biochemical study. *Contemporary Clinical Dentistry*, 2011. 2(4), 278.
136. Kumar AK, Reddy NR, Babu M, Kumar PM, Reddy VS, Chavan CV. Estimation of prostaglandin E2 levels in gingival crevicular fluid in periodontal health, disease and after treatment. *Contemporary Clinical Dentistry*, 2013; 4(3), 303.
137. Kurdukar PA, Kurdukar AA, Mahale SA, Beldar AM. Biomarkers in gingival crevicular fluid. *IOSR Journal of Dental and Medical Sciences*, 2015; 14(10), 104-109.
138. Lalla E, Cheng B, Lal S, Kaplan S, Softness B, Greenberg E, Goland RS, Lamster IB: Diabetes mellitus promotes periodontal destruction in children. *J Clin Periodontol* 2007; 34: 294–298.

139. Lalla E, Cheng B, Lal S, Kaplan S, Softness B, Greenberg E, Goland RS, Lamster IB: Diabetes- related parameters and periodontal conditions in children. *J Periodontal Res* 2007; 42: 345–349
140. Lalla E, Cheng B, Lal S, Tucker S, Greenberg E, Goland R, Lamster IB: Periodontal changes in children and adolescents with diabetes: a case-control study. *Diabetes Care* 2006; 29:295–299.
141. Lamster IB, Ahlo JK. Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Annals of the New York Academy of Sciences*, 2007; 1098(1): 216-229.
142. Lamster IB. The host response in gingival crevicular fluid: potential applications in periodontitis clinical trials. *Journal of Periodontology*, 1992. *63*, 1117-1123.
143. Langston H, Ke Y, Gewirtz A, Dombrowski K, Kapp J. Secretion of IL-2 and IFN-y, but not IL-4, by antigen-specific T cells requires extracellular ATP. *J Immunol*, 2003; 170:2962–70.
144. Lejeune J, Gautier M, Turpin R, Les chromosomes humains en culture de tissus, C.R. Acad. Sciences, Paris, 1959, 26.
145. Leppilahti JM, Hernández-Ríos PA, Gamonal JA, Tervahartiala T, Brignardello-Petersen R, Mantyla P, Hernández M. Matrix metalloproteinases and myeloperoxidase in gingival crevicular fluid provide site specific diagnostic value for chronic periodontitis. *Journal of Clinical Periodontology*, 2014; 41(4), 348-356.
146. Linden GJ, Lyons A, Scannapieco FA. Periodontal systemic associations: review of the evidence. *Journal of Periodontology*; 2013; 84: S8-S19.
147. Littarru GP, Tiano L. Clinical aspects of coenzyme Q10: an update. *Nutrition.* 2010; 26:250-411.
148. Lopes IA, Nogueira DN. Oral manifestations of chemotherapy in children. *Pesqui bras pediatric dentistry clinic integral* 2012; 12(1): 113-119.
149. López-Pérez R, Borges-Yáñez SA, Jiménez-García G, Maupomé G. Oral hygiene, gingivitis, and periodontitis in persons with Down syndrome. *Spec Care Dentist*. 2002;22(6):214-220.
150. Luchian I, Martu I, Goriuc A, Vata I, Hurjui L, Matei MN, Martu S. Salivary PGE2 as a Potential Biochemical Marker during Orthodontic Treatment Associated with Periodontal Disease. *Rev. Chim. (Bucharest).* 2016; 67(10): 2119-2123.
151. Luchian I, Martu I, Ioanid N, Goriuc A, Vata I, Hurjui L, Martu-Stefanache A, Tatarciuc M, Matei MN, Martu S. Salivary IL-1β: A Biochemical Marker that Predicts Periodontal Disease in Orthodontic Treatment. *Rev. Chim. (Bucharest).* 2016; 67(12): 2749-2483.
152. Lynch MA, Ship II. Initial oral manifestations of leukemia. *JADA* 1967;75(4):932-940.
153. Maftei GA, Toma V, Filioreanu AM, Ciurcanu O, Popa C, Foia L. A case of bilateral impaction of maxillary incisors in an intellectually challenged patient. *Romanian Journal of Medical and Dental Education*, 2018; 7(2): 81-86.
154. Magrone T, Jirillo E. Childhood obesity: immune response and nutritional approaches. *Front Immunol.* 2015;6:76.23.

155. Mäntylä P, Stenman M, Kinane DF, Tikanoja S, Luoto H, Salo T, Sorsa T. Gingival crevicular fluid collagenase 2 (MMP8) test stick for chairside monitoring of periodontitis. *Journal of Periodontal Research*, 2003; 38(4): 436-439.
156. Mares M, Minea B, Nastasa V, Rosca I, Bostanaru AC, Marincu I, Toma V, Cristea VC, Murariu C, Pinteala M. In vitro activity of echinocandins against 562 clinical yeast isolates from a Romanian multicentre study. *Medical Mycology*, 2018; 56(4): 442-451.
157. Martínez-Cué C, Delatour B, Potier MC. Treating enhanced GABAergic inhibition in Down syndrome: use of GABA5-selective inverse agonists. *Neurosci Biobehav Rev.* 2014; 46:218-273.
158. Matičičr M, Poljak M, Kramar B, Seme K, Brinovec V, Meglič-Volkar J, Skalerič, U. Detection of hepatitis C virus RNA from gingival crevicular fluid and its relation to virus presence in saliva. *Journal of Periodontology*, 2001; 72(1): 11-16.
159. Matthews DC, The relationship between diabetes and periodontal disease. *Journal of the Canadian Dental Association* 2002; vol. 68, no. 3, p. 161–164.
160. Mattson MP, Chan SL. Calcium orchestrates apoptosis. *Nature Cell Biol.* 2003, 5(12): 1041-1043.
161. Maxim A, Savin C, Bălan A, Păsăreanu M, Toma V, Maxim DC, Petcu A, Serban V. The impact of socio-cultural model on the child education for oral health. *Romanian Journal of Oral Rehabilitation*, 2011; 3(1): 43-47.
162. McCord C, Johnson L. Oral manifestations of hematologic disease. *Atlas Oral Maxillofacial Surg Clin N Am.* 2017; 1061-3315/17.
163. McKenna SJ. Leukemia. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2000; 89(2): 137-9.
164. McKenzie WT, Forgas L, Vernino AR, Parker D, Limestall JD. Comparison of a 0.12% chlorhexidine mouthrinse and an essential oil mouthrinse on oral health in institutionalized, mentally handicapped adults: One year results. *J Periodontol.* 1992; 63: 187–193.
165. Mealey BL, Oates TW: Diabetes mellitus and periodontal diseases. *J Periodontol.* 2006; 77: 1289–1303.
166. Mealey BL, Rose LF. Diabetes mellitus and inflammatory periodontal diseases. *Compend Contin Educ Dent.* 2008; 29(7):402-8,410,412-3.
167. Mescua NG, Rodrigues MM, Zotarelli-Filho IJ, Tempest LM. Dental care for children with Leukemia: Major updates. *Int J Dentistry Oral Sci.* 2017; 4(11): 546-551.
168. Meurman JH. Translational Oral Health Research. Springer 2018. ISBN 978-3-319-78204-1. p.1-33.
169. Meyle J, Gonzales JR. Influences of systemic diseases on periodontitis in children and adolescents. *Periodontol 2000.* 2001; 26: 92–112.
170. Mi QS, Ly D, Zucker P, McGarry M, Delovitch T. Interleukin-4 but not Interleukin-10 Protects Against Spontaneous and Recurrent Type 1 Diabetes by Activated CD1d-Restricted Invariant Natural Killer T-Cells. *Diabetes* 2004; 53(5): 1303-1310.

171. Michaud M, Baehner RL, Bixler D, Kafrawy AH. Oral manifestations of acute leukemia in children. *J Am Dent Assoc*. 1977; 95(6):1145-50.
172. Miles MV, Patterson BJ, Chalfonte-Evans ML, Horn PS, Hickey FJ, Schapiro MB, et al. Coenzyme Q10 (ubiquinol-10) supplementation improves oxidative imbalance in children with trisomy21. *Pediatr Neurol.* 2007;37:398-403.7.
173. Mishra MB, Khan ZY, Mishra S. Gingival overgrowth and drug association: a review. *Indian J Med Sci,* 2011, 65(2): 73–82.
174. Moffitt ML, Bencivenni D, Cohen RE. Drug-induced gingival enlargement: an overview. *Compend Contin Educ Dent,* 2013, 34(5):330–336.
175. Monisha K., Savitha G. Assessment of Oxidative Stress in Periodontitis Patients. *J Pharm. Sci. & Res.* 2016; Vol 8(7), 620-622.
176. Morais EF, Lira JA, Macedo RA, Santos KS, Elias CT, Morais Mde L. Oral manifestations resulting from chemotherapy in children with acute lymphoblastic leukemia. *Braz J Otorhinolaryngol*. 2014; 80:78-85.
177. Morgan J. Why is periodontal disease more prevalent and more severe in people with Down syndrome? Special Care in Dentistry 2007; 27(5): 196-201.
178. Morozumi T, Kubota T, Sato T, Okuda K, Yoshie H. Smoking cessation increases gingival blood flow and gingival crevicular fluid. *Journal of Clinical Periodontology*, 2004. 31(4), 267-272.
179. Nakhostin A, Meighani G. Leukemia and its oral manifestation in children younger than 12 years reffered to Tehran pediatric hospitals. *Avicenna J Dent Res.* 2016; 8(4): e29995.
180. Nakib N, Ashrafi S. Drug-induced gingival overgrowth. *Dis Mon,* 2011, 57(4):225–230.
181. Nănescu S, Mârțu S, Ciomaga G, Toma Vasilica, Forna D, Foia L. Dual effects of flavonoids on dyslipidemia and periodontal disease. *Romanian Journal of Oral Rehabilitation*, 2011; 3(4):38-45.
182. Nănescu S., Mârțu S., Constantin L. Activitatea antioxidantă redusă în boala parodontală. *Journal of Romanian Dentistry,* 2006; 10: 1.
183. Nishimura F, Iwamoto Y, Soga Y: The periodontal host response with diabetes. *Periodontol 2000*. 2007; 43: 245–253.
184. Nizri E, Brenner T. Modulation on inflammatory pathways by the immune cholinergic system. *Amino Acids* 2013; 45:73–85.
185. Nizri E, Hamra-Amitay Y, Sicsic C, Lavon I, Brenner T. Anti-inflammatory properties of cholinergic up-regulation: a new role for acetylcholinesterase inhibitors. *Neuropharmacology* 2006; 50:540–7.
186. Noda, D., Hamachi, T., Inoue, K. & Maeda, K. (2007). Relationship between the presence of periodontopathic bacteria and the expression of chemokine receptor mRNA in inflamed gingival tissues, *J Periodontal Res.* 42(6): 566-71.
187. Novotna M, Stepan P, Zdenek B, Erika Lencova, and Jana Duskova. Periodontal Diseases and Dental Caries in Children with Type 1 Diabetes Mellitus. *Mediators of Inflammation*, vol. 2015, Article ID 379626, 8 pages, 2015. https://doi.org/10.1155/2015/379626.

188. Opal SM, DePalo VA. Anti-inflammatory cytokines. *Chest 2000*; 117:1162–72.
189. Orner G, Periodontal disease among children with Down's syndrome and their siblings. *Journal of Dental Research* 1976; 55: 778-782.
190. Oswal S, Dwarakanath CD. Relevance of gingival crevice fluid components in assessment of periodontal disease – A critical analysis. *Journal of Indian Society of Periodontology*, 2010; 14(4): 282.
191. Park KS, Kim TK, Kim DH. Cyclosporine A treatment alter characteristic of Ca2+-release channel in sarcoplasmic reticulum. *Am. J. Periodontol.* 1999, 4 (12):154-159.
192. Park MJ, Sotomatsu M, Ohki K, Arai K, Maruyama K, Kobayashi T. et al. Liver disease is frequently observed in Down syndrome patients with transient abnormal myelopoiesis. *Int J Hematol*. 2014; 99: 154–161.
193. Parodi A, Battaglia F, Kalli F, Ferrera F, Conteduca G, Tardito S, Stringara S, Ivaldi F, Negrini S, Borgonovo G, Simonato A, Traverso P, Carmignani G, Fenoglio D, Filaci G. CD39 is highly involved in mediating the suppression activity of tumor infiltrating CD8+ T regulatory lymphocytes. *Cancer Immunol Immunother* 2013; 62:851–62.
194. Patrick DL, Lee RSY, Nucci M, Grembowski D, Jolles CZ, Milgrom P. Reducing Oral Health Disparities: A Focus on Social and Cultural Determinants. *BMC Oral Health*. 2006; 6(Suppl 1): 1–17.
195. Pels E, Comparison of saliva interleukin-2 concentration to the condition of gums in children with acute lymphoblastic leukaemia during anti-tumour treatment. *Cancer Chemother Pharmacol,* 2015; 76:205-210.
196. Pels E, Mielnik-Blaszczak M. Oral hygiene in children suffering from acute lymphoblastic leukemia living in rural and urban regions. *Ann Agric Environ Med*. 2012; 19(3): 529-533.
197. Perluigi M, Butterfield DA. Oxidative stress and Down Syndrome: a route toward Alzheimer-like dementia. *Curr Gerontol GeriatrRes*. 2012; 2012:724904.5.
198. Perluigi M, Pupo G, Tramutola A, Cini C, Coccia R, Barone E,et al. Neuropathological role of PI3K/Akt/mTOR axis in Down syndrome brain. *Biochim Biophys Acta.* 2014; 1842:1144-1153.
199. Philipone E, Yoon AJ. Oral Pathology in the Pediatric Patient. A clinical guide to the Diagnosis and Treatment of Mucosal Lesions. *Springer* 2017; 10:129.
200. Phillis JW. Adenosine and adenine nucleotides as regulators of cellular function. *Boca Raton, Florida, U.S.A.: CRC press*; 1991; 1st ed.
201. Pihlstrom, BL., Michalowicz, BS. & Johnson, NW. Periodontal diseases. *Lancet.* 2005; 19: 1809-20.
202. Pinheiro DS, Souza AD, Albuquerque de JS. Infant mortality for lymphoid leukemia in Brazil regions. *Brazil J Technol Innov Health*, 2016; 6(1):11-21.
203. Pinto LP., Batista de Souza L., Gordon-Nunez MA., Calasanz Soarez R., Brito Costa EM., Luz de Aquino AR., Fernandes MZ. Prevention of oral lesions in children with acute lymphoblastic leukemia. *International Journal of Pediatric Otorhinolaryngology* 2006; 70:1847-1851.

204. Pizzo G, Guiglia R, Russo LL, Campisi G. Dentistry and internal medicine: from the focal infection theory to the periodontal medicine concept. *European Journal of Internal Medicine*, 2010. 21(6), 496-502.
205. Pöllänen M T, Salonen JI. Uitto VJ. Structure and function of the tooth–epithelial interface in health and disease. *Periodontology 2000*, 2003. 31(1), 12-31.
206. Pourshari WP, Saghiri R, Ebrahimi-Rad M, et al. Adenosine deaminase in patients with primary immunodeficiency syndromes: the analysis of serum ADA1 and ADA2 activities. *Clin Biochem* 2009; 42:1438–43.
207. Pradeep AR, Prapulla DV, Sharma A, Sujatha PB. Gingival crevicular fluid and serum vascular endothelial growth factor: their relationship in periodontal health, disease and after treatment. *Cytokine*, 2011; 54(2): 200-204.
208. Preshaw PM, Alba AL, Herrera D, et al. Periodontitis and diabetes: a two-way relationship. *Diabetologia*, 2012; 55(1):21-31.
209. Preshaw PM, Taylor JJ. How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis? *Journal of Clinical Periodontology*, 2011; 38, 60-84.
210. Puukka R, Puukka M, Linna S, Leppilampi M, Kouvalainen K. Erythrocyte adenosine deaminase, purine nucleoside phosphorylase and phosphoribosyl transferase activity in patients with Down's syndrome. *Clin Chim Acta* 1982; 126:275–81.
211. Racovita S, Bunia I, Plesca I, Vasiliu S, Profire L, Foia L, Toma V. Release studies of cefotaxime sodium salt from coated ion exchange resin microparticles. *Farmacia.* 2017; 65(6):832-836.
212. Ram G, Chinen J. Infections and immunodeficiency in Down syndrome. Clin Exp Immunol 2011; 164:9–16.
213. Rasmussen L, Hänström L, Lerner UH. Characterization of bone resorbing activity in gingival crevicular fluid from patients with periodontitis. *Journal of Clinical Periodontology*, 2000; 27(1), 41-52.
214. Rasool M., Farooq S., Malik A., Shaukat A., Manan A., Asif M., Sani S., Qazi M, Kamal M., Iqbal Z., Hussain A. Assessment of circulating biochemical markers and antioxidative status in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients. *Saudi Journal of Biological Sciences,* 2015; 22: 106-111.
215. Rateitschak-Plüss EM, Hefti A, Rateitschak KH. Gingival hyperplasia from cyclosporine A medication. *SSO Schweiz Monatsschr Zahnheilkd,* 1983; 93(1):57–65.
216. Reardon C, Duncan GS, Brüstle A, Brennera D. Lymphocyte-derived ACh regulates local innate but not adaptive immunity. *Proc Natl Acad Sci U S A* 2013; 110:1410–5.
217. Riond J., Riviere J. Pharmacology and toxicology of doxycycline. *Vet Hum Toxicol*. 1988; 30(5):431-43.
218. Roath S, Israuels MC, Wilkinson JF. The acute leukaemias: a study of 580 patients. *Q J Med*. 1964; 33:256-283.

219. Robertson, RP. & Harmon, JS. Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet beta cell, *Free Rad. Biol. Med.* 2006; 41(2): 177-184.
220. Rodrigues R, Debom G, Soares F, Machado C, Pureza J,Peres W, et al. Alterations of ectonucleotidases and acetyl-cholinesterase activities in lymphocytes of Down syndrome subjects: relation with inflammatory parameters. *Clin Chim Acta.* 2014; 433:105-114.
221. Rostami MN, Douraghi M, Mohammadi AM, Nikmanesh B. Altered serum pro-inflammatory cytokines in children with Down's syndrome. *Eur Cytokine Netw*. 2012; 23: 64–67.
222. Rowley WR, Bezold C, Arikan Y, Byrne E, Krohe S. Diabetes 2030: insights from yesterday, today, and future trends. *Popul Health Manag.* 2017; 20(1):6-12.
223. Ryan M. E., Carnu O., Kamer A. The influence of diabetes on the periodontal tissues. *Journal of the American Dental Association* 2003; vol. 134, pp. 34–40.
224. Safari A, Kompani F, Memarian S, Monajemzadeh M, Khosravi R, Gharib B. A 9-year old boy with leukemia and oral manifestation: a case report and review of literature. *Galen Medical Journal* 2016; 5(4):215-18.
225. Sakellari D, Belibasakis G, Chadjipadelis T, Arapostathis K, Konstantinidis A. Supragingival and subgingival microbiota of adult patients with Down's syndrome. Changes after periodontal treatment. *Oral Microbiol Immunol.* 2001; 16: 376–382.
226. Salvi GE, Franco LM, Braun TM, Lee A, Rutger Persson G, Lang NP, et al. Pro-inflammatory biomarkers during experimental gingivitis in patients with type 1 diabetes mellitus: a proof-of-concept study. *J Clin Periodontol.* 2010; 37(1):9-16.
227. Salvi GE, Kandylaki M, Troendle A, Persson GR, Lang NP: Experimental gingivitis in type 1 diabetics: a controlled clinical and microbiological study. *J Clin Periodontol.* 2005; 32: 310–316.
228. Samprasit W., Kaomongkolgit R., Sukma M., Rojanarata T., Ngawhirunpat T., Opanasopit P. Mucoadhesive electrospun chitosan-based nanofibre mats for dental caries prevention. *Carbohydr Polym.* 2015; Mar 6;117:933-40.
229. Sapna G, Gokul S, BagriManjrekar K. Matrix metalloproteinases and periodontal diseases. *Oral diseases*, 2014; 20(6), 538-550.
230. Schenkein HA, Loos BG. Inflammatory mechanisms linking periodontal diseases to cardiovascular diseases. *Journal of Clinical Periodontology*, 2013; 40: S51-S69.
231. Schlosser BJ, Pirigyi M, Mirowski GW. Oral manifestations of hematologic and nutritional diseases. *Otolaryngol Clin N Am*. 2011(44): 183-203.
232. Séguin E, Traitement Moral, Hygiène et éducation des idiots et des autres enfants arriérés, Baillière, Paris, 1846.
233. Seymour RA, Smith DG. The effect of a plaque control programme on the incidence and severity of cyclosporine-induced gingival changes. *J Clin Periodontol.* 1991; 18(2): 107–110.
234. Shimada K, Mizuno T, Ohshio K, Kamaga M, Murai S, Ito K. Analysis of aspartate aminotransferase in gingival crevicular fluid assessed by using PocketWatchTM: a

longitudinal study with initial therapy. *Journal of Clinical Periodontology*, 2000. 27(11): 819-823.

235. Shukla D, Bablani D, Chowdhry A, Thapar R, Gupta P, MishraS. Dentofacial and cranial changes in Down syndrome. *Osong Public Health Res Perspect.* 2014;5:339-44.27.
236. Shyama M, Al-Mutawa SA, Honkala S, Honkala E. Supervised tooth brushing and oral health education program in Kuwait for children and young adults with Down syndrome. *Spec Care Dent.* 2003; 23: 94–99.
237. Sies, H. Oxidative stress: oxidants and antioxidants. *Experimental Physiology.*1997; 82, 291–295.
238. Silness, J & Löe, H. Periodontal disease in pregnancy II. Correlation between oral hygiene and periodontal condition. *Acta Odontol Scand* 1964; 24: 121-135.
239. Silvestri L. The regulation of intracellular calcium ion concentration and mitochondrial function by cyclosporine A: a putative mechanism for the pathogenesis of gingival overgrowth. *PhD Thesis*, 2000; Univ. of Toronto.
240. Simmons, RA. Developmental origins of diabetes: The role of oxidative stress, *Free Rad. Biol. Med.* 2006; 40(6): 917–922.
241. Smith C.E., Hu Y., Richardson A.S., Bartlett J.D., Hu J.CC., Simmer J.P. Relationships between protein and mineral during enamel development in normal and genetically altered mice. *Eur J Oral Sci.* 2011; 119(Suppl 1): 125–135.
242. Sorsa T, Suomalainen K. Uitto VJ. The role of gingival crevicular fluid and salivary interstitial collagenases in human periodontal diseases. *Archives of Oral Biology*, 1990. 35, S193-S196.
243. Spanevello RM, Mazzanti CM, Bagatini M, et al. Activities of the enzymes that hydrolyse adenine nucleotides in platelets from multiples sclerosis patients. *J Neurol* 2010; 257:24–30.
244. Stabholz A, Shapira J, Shur D, Friedman M, Guberman R, Sela MN. Local application of sustainedrelease delivery system of chlorhexidine in Down's syndrome population. *Clin Prev Dent.* 1991; 13: 9–14.
245. Stafford R, Sonis S, Lockhart P, Sonis A. Oral pathoses as diagnostic indicators in leukemia. *Oral Surg Oral Med Oral Pathol*. 1980; 50(2):134-139.
246. Steliarova-Foucher E, Stiller C, Kaatsch P, Berrino F, Coebergh JW, Lacour B, Parkin M. Geographical patterns and time trends of cancer incidence and survival among children and adolescents in Europe since the 1970s (the ACCIS project): an epidemiological study. *Lancet* 2004; 364: 2097-2105.
247. Sujoy G, Feingold E, and Subrata KD, Am. J. Med. Genet. A, 149A(7) (2009) 1415-1420.
248. Suppipat N. Evaluation of an electronic device for gingival fluid quantitation. *Journal of Periodontology*, 1977. 48(7): 388-394.
249. Şurlin P, Rauten AM, Mateescu GO, Oprea B, Mariş M, Manolea H. The involvement of metalloproteinases and their tissular inhibitors in the processes of periodontal ortho-dontic remodeling. *Rom J Morphol Embryol*, 2009, 50(2): 181–184.

250. Şurlin P, Rauten AM, Mogoantă L, Siloşi I, Oprea B, Pirici D. Correlations between the gingival crevicular fluid MMP8 levels and gingival overgrowth in patients with fixed ortho-dontic devices. *Rom J Morphol Embryol*, 2010, 51(3):515– 519.
251. Şurlin P, Rauten AM, Pirici D, Oprea B, Mogoantă L, Camen A. Collagen IV and MMP-9 expression in hypertrophic gingiva during orthodontic treatment. *Rom J Morphol Embryol,* 2012, 53(1):161–165.
252. Surna A, Kubilius, R, Sakalauskiene J, Vitkauskiene A, Jonaitis J, Saferis V, Gleiznys A. (2009). Lysozyme and microbiota in relation to gingivitis and periodontitis. *Medical Science Monitor*, 15(2), CR66-CR73.
253. Suzuki T, Omata K, Satoh T, Miyasaka T, Arai C, Maeda M, Miyamura T. Quantitative detection of hepatitis C virus (HCV) RNA in saliva and gingival crevicular fluid of HCV-infected patients. *Journal of Clinical Microbiology*, 2005; 43(9): 4413-4417.
254. Sznajder N, Carraro JJ, Otero E, Carranza FA jr. Clinical periodontal findings in trisomy 21 (mongolism), *Journal of Periodontal Research*, 1968; 3: 1-5.
255. Tabholz, A., Soskolne, WA. & Shapira, L. Genetic and environmental risk factors for chronic periodontitis and aggressive periodontitis, *Periodontol 2000.* 2010; 53: 138-153.
256. Takagi M, Sakota Y, Ishikawa G, Kamiyama R, NakajimaT, Nomura T. Oral manifestations of acute promyelocytic leukemia. *J Oral Surg*. 1978; 36(8):589-593.
257. Tanaka M, Giro E, Cavalcante L, et al. Expression of interferon γ e interferon α and related genes individuals with down syndrome and periodontitis. *Cytokine* 2012; 60:875–81.
258. Taylor GW, Borgnakke WS: Periodontal disease: associations with diabetes, glycemic control and complications. *Oral Dis* 2008; 14: 191–203.
259. Teles R, Sakellari D, Teles F, Konstantinidis A, Kent R, Socransky S, Haffajee A. Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. *Journal of Periodontology*, 2010. *81*(1), 89-98.
260. Thomason JM, Seymour RA, Rice N. The prevalence and severity of cyclosporine and nifedipine-induced gingival overgrowth. *J Clin Periodontol*, 1993, 20(1):37–40.
261. Thompson AL, Herman WW, Konzelman J, Collins MA. Treating patients with drug-induced gingival overgrowth. *J Dent Hyg*, 2004, 78(4):12.
262. Tiano L, Busciglio J. Mitochondrial dysfunction and Down's syndrome: is there a role for coenzyme Q10? *Biofactors.* 2011; 37:386-392.
263. Tiano L, Padella L, Santoro L, Carnevali P, Principi F, BrugèF, et al. Prolonged coenzyme Q10 treatment in Down syndrome patients: effect on DNA oxidation. *Neurobiol Aging.* 2012; 33:626, e1---8.6.
264. Tilakaratne A., Soory M., Antioxidant response of osteoblasts to doxycycline in an inflammatory model induced by C-reactive protein and interleukin-6. *Infect Disord Drug Targets* 2014; 14(1):14-22.
265. Tiranathanagul S, Yongchaitrakul T, Pattamapun K, Pavasant P. Actinobacillus actinomycetemcomitans lipopolysaccharide activates matrix metalloporteinase-2 and

increases receptor activator of nuclear factor-kappaB ligand expression in human periodontal ligament cells. *J Periodontol*. 2004;75(12):1647-1654.

266. Toma V, Adumitroaie A, Cioloca D, Golovcencu L, Foia L, Halitchi LG, Zegan G, Gingivo-periodontal manifestations in children with leukemia: a literature review. *The Medical-Surgical Journal*, 2019.
267. Toma V, Cioloca DP, Forna DA, Hurjui L, Botnariu G, Nechifor IE, Bogdan M, Costuleanu M, Simion L, Holban C. IL-18 as an important gingival inflammatory biochemical marker in children and adolescents with insulin-dependent diabetes mellitus. *Rev Chim*. 2016; 67(12):2545-2551.
268. Toma V, Foia LG, Forna D, Toma CM, Cioloca D, Balan G, Balan A. Down Syndrome between genetic hazard and divine decision. *European Journal of Science and Theology*, 2014; 10(3): 49-59.
269. Toma V, Goriuc A, Cioloca D, Kozma A, Iordache C, Topoliceanu C, Filip F, Gamen A, Zegan G. Evaluation of periodontal status in a group of children affected by Down Syndrome. *Romanian Journal of Medical and Dental Education*, 2019;8(3): 28-34.
270. Toma V, Goriuc A, Cioloca D, Nechifor I, Surdu A, Maftei G, Foia L, Filip F. The role of malondialdehyde in evaluation of the oxidative status of periodontal impairment. *Romanian Journal of Medical and Dental Education*, 2017; 6(1): 6-14.
271. Toma V, Surlin P, Cioloca D, Trandafir L, Cozma S, Bogdan M, Botnariu EG, Balan A. Assessment of clinical periodontal modifications in juvenile diabetes. Romanian Journal of Oral Rehabilitation, 2015; 7(2): 97-101.
272. Toma V, Surlin P, Cioloca D, Trandafir L, Cozma S, Bogdan M, Botnariu EG, Balan A. Assessment of clinical periodontal modifications in juvenile diabetes. *Romanian Journal of Oral Rehabilitation*, 2015; 7(2): 97-101.
273. Toma V, Maxim A, Balan A, Gheban D, Rotaru DC, Filip F, Foia L. Periodontal aspects in children and adolescents with Down Syndrome. *Romanian Journal of Oral Rehabilitation*, 2009; 1(4): 35-42.
274. Tonetti MS, Claffey N; European Workshop in Periodontology group C. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *J Clin Periodontol.* 2005; 32 Suppl 6:210-213.
275. Topoliceanu C, Ghiorghe CA, Toma V, Andrian S, Iovan G, Stoleriu S, Pancu G, Nica I, Salceanu M. Assessment of Near-Infrared (780 nm) Light Transillumination Accuracy for detectection of incipient caries lesions. *Rev Chim*. 2019; 70(4): 1333-1336.
276. Trotta MBF, Azul JBS, Wajngarten M, Fonseca SG, Goldberg AC, Kalil JE. Inflammatory and Immunological parameters in adults with Down syndrome. *Immun Ageing* 2011; 8:4.
277. Uitto VJ. (2003). Gingival crevice fluid–an introduction. *Periodontology 2000*, 31(1), 9-11.

278. Valera MC., Noirrit-Esclassan E., Pasquet M., Vaysse F. Oral complications and dental care in children with acute lymphoblastic leukaemia. *J Oral Pathol Med* 2015; 44:483-489.
279. Veisa G, Donciu MD, Segal L, Hurjui L, Nistor I, Ursarescu IG, Martu S, Burlea L, Solomon S. Albumin as a prognostic factor for malnutrition and inflammation in chronic kidney disease. *Rev. Chim. (Bucharest).* 2016; 67(1):103-105.
280. Verstegen R, Kusters M, Gemen E, Vries E. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. *Pediatr Res* 2010; 67:563–569.
281. Vlad CE, Foia L, Agache CA, Strungaru SA, Toma V, Surdu A, Goriuc A, Florea L. Decompensated diabetes mellitus binomial – emphysematous pyelonephritis and periodontal disease. *Romanian Journal of Oral Rehabilitation.* 2017; 9(4): 97-104.
282. Vlad CE, Foia L, Agache CA, Strungaru SA, Toma V, Surdu A, Goriuc A, Florea L. Decompensated diabetes mellitus binomial – emphysematous pyelonephritis and periodontal disease. *Romanian Journal of Oral Rehabilitation*. 2017; 9(4): 97-104.
283. Vural F, Dzcan MA. Gingival involvment in patient with CD56+, chronic myelomonocytic leukemia. *Leuk lymphoma* 2004; 4:415-18.
284. Wang X. The expending role of mitochondria in apoptosis. *PhD Thesis*, 2003. Howard Hughes Medical Institute and Department of Biochemistry, Texas.
285. Wang Y., Xue J., Zhou X., You M., Du Q., Yang X., He J., Zou J., Cheng L., Li M., Li Y., Zhu Y., Li J., Shi W., Xu X. Oral microbiota distinguishes acute lymphoblastic leukemia pediatric hosts from healthy populations. *PLoS ONE,* 2014; 9(7).
286. Wassall RR, Preshaw PM. Clinical and technical considerations in the analysis of gingival crevicular fluid. *Periodontology 2000*, 2016; 70(1): 65-79.
287. Wong R, Steenbergen C, Murphy E. Mitochondrial permeability transition pore and calcium handling. *Methods Mol Biol.* 2012; 810: 235-242.
288. Wooton AK, Melchior LM, Coan LL, Reddington AR. Periodontal disease in children with type 2 diabetes mellitus. *Nurse Pract.* 2018;43(2):30-35.
289. Xu Y, Li W, Liu X, Chen H, Tan K, Chen Y, Tu Z, Dai Y. Identification of dysregulated microRNAs in lymphocytes from children with Down syndrome. *Gene* 2013;10:278–86.
290. Yu GY, He G, Li CY, Tang M, Grivennikov S, Tsai WT, Karin, M. Hepatic expression of HCV RNA-dependent RNA polymerase triggers innate immune signaling and cytokine production. *Molecular Cell*, 2012; 48(2): 313-321.
291. Zaldivar-Chiapa RM, Arce-Mendoza AY, De La Rosa-Ramírez M, Caffesse RG, Solis-Soto JM. Evaluation of Surgical and Non-Surgical Periodontal Therapies, and Immunological Status, of Young Down's Syndrome Patients. *J Periodontol.* 2005; 76: 1061–1065.
292. Zampieri BL, Biselli-Périco JM, de Souza JES, Bürger MC, Silva WA Jr, Goloni-Bertollo EM, et al. Altered expression of immune-related genes in children with Down syndrome. *PLoS One.*2014; 9:1–6.

293. Zandbergen D, Slot DE, Cobb CM, Van der Weijden FA. The clinical effect of scaling and root planning and the concomitant administration of systemic amoxicillin and metronidazole: a systematic review. *J Periodontol.* 2013; 84: 332–351.
294. Zebisch M, Krauss M, Schäfer P, Lauble P, Sträter N. Crystallographic snapshots along the reaction pathway of nucleoside triphosphate diphosphohydrolases. *Structure* 2013; 21:1460–75.
295. Zegan G, Anistoroaei D, Cernei ER, Sodor A, Toma V. Assessment of schoolchildrens' knowledge, attitudes and behaviors for oral health. *Romanian Journal of Medical and Dental Education*, 2019; 8(2):64-72.
296. Zegan G, Anistoroaei D, Dascalu CG, Cernei ER, Toma V. Morphological aspect and surface chemical analysis of salivary crystals in young patients with/without orthodontic appliances, *International Journal of Medical Dentistry*, 2019; 23(1):p 57-63.
297. Zegan G, Toma V, Cernei ER, Anistoroaei D, Carausu EM, Moscu M. Study on Antibiotic Loaded Nanoparticles for Oral Infection Treatment, *Rev Chim*. 2019; 70(5); 1712-1714.
298. Zegan G., Carausu E.M., Golovcencu L., Botezatu-Sodor, A., Cernei E.R., Anistoroaei D. Antibiotic-anionic clay matrix used for drug controlled release. *Rev. Chim.(Bucharest)*, 69, no. 2, 2018, p. 321.
299. Zegan G., Cernei E.R., Carausu E.M., Golovcencu L., Anistoroaei D. Structural characteristics of drug intercalated hydrotalcites used in dental medicine. *Rev. Chim.(Bucharest)*, 70, no. 4, 2019, p. 1215.
300. Zhou J, Windsor LJ. Porphyromonas gingivalis affects host collagen degradation by affecting expression, activation, and inhibition of matrix metaloproteinases. *J Periodontal Res*. 2006;41(1):47-54.
301. ***Centers for Disease Control and Prevention. *Diabetes/Healthy People 2020*. 2016. www.healthypeople.gov/2020/topics-objectives/topic/diabetes