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**METHODS FOR IDENTIFYING, EVALUATING AND
MONITORING THE EFFECTS OF PERIPHERAL
INFLAMMATION AND DYSBIOSIS FROM ORAL REGION**

ABSTRACT

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TABLE OF CONTENTS

Lists of published papers	VI
Abbreviations	VII
List of figures	X
List of tables	XI
Dedication.....	XII
Special thanks	XIII
INTRODUCTION	XV

GENERAL PART

1. Periodontal disease	1
1.1. Definition and diagnosis	1
1.2. Epidemiological data	2
1.3. Classification of the periodontal disease	3
1.4. Conditions affecting the periodontium	8
1.5. Risk elements in etiology of periodontal disease	9
2. Alzheimer's disease	11
2.1. Definition and diagnosis	11
2.2. Epidemiology	12
2.3. Classification of Alzheimer's disease	13
2.4. Risk factors in Alzheimer's disease	15
3. The link between periodontal and Alzheimer's disease	16
3.1. Inflammatory mechanism	16
3.2. Infectious mechanism	19
4. Biomarkers in Alzheimer's disease	21
4.1. Salivary microbiome	22
4.2. Salivary metabolites	23

SPECIFIC PART

5. Periodontal disease in a young romanian convenience sample:	
radiographic assessment	26
5.1. Introduction.....	26
5.2. Material and methods	27
5.3. Statistical analysis	32
5.4. Results	34
5.4.1. Prevalence of periodontal disease	36
5.4.2. Characteristics of the subjects by periodontal condition	36
5.4.3. Periodontal conditions associate with age, tooth number and family history 39	
5.5. Discussion.....	41
5.6. Conclusions.....	44
6. Cognitive dysfunction in young subjects with periodontal disease.....	45
6.1. Introduction.....	45
6.2. Methods and materials	46
6.2.1. Inclusion criteria.....	47
6.2.2. Outcome measure.....	47
6.2.3. Clinical assessments	48
6.2.4. Neuropsychological assessment	48
6.2.5. Saliva collection and cytokine assessment	50
6.2.6. Elisa-based cytokine analysis from saliva samples	50
6.2.7. Statistical methods	52
6.3. Results	53
6.3.1. Delayed recall and immediate recall scores were lower in subjects with periodontitis	55
6.3.2. Learning curves differed among the periodontal groups	58
6.3.3. Salivary IL-1 β but not TNF- α , associated with immediate cognitive score.....	60
6.4. Discussion.....	61
6.4.1. Strengths and weaknesses	63
6.5. Conclusions.....	64
7. Targeted salivary metabolomic profiling in patients with different stages of periodontitis using chip-based high-resolution mass spectrometry.....	65

7.1. Introduction.....	65
7.2. Methods and materials.....	68
7.2.1. Study population	68
7.2.2. Sample collection and processing	69
7.2.3. Sample preparation	70
7.2.4. Chip-based nanoESI-qTOF MS analysis	71
7.3. Results	72
7.4. Discussions	85
7.5. Conclusion	89
8. CONCLUSIONS AND PERSONAL CONTRIBUTIONS	90
BIBLIOGRAPHY:.....	94
ANNEXES	I

Key words: periodontal disease, Alzheimer's disease, inflammation, infection

INTRODUCTION

Periodontal disease (PD), represents both an infectious and an inflammatory condition and its worldwide prevalence confers to the Global Burden of Disease Study from 2016 as the 11th uttermost prevailing condition, ranging from 20- 50% and increases with age from adolescence to adult to mature society.

On the other hand, according to the World Health Organization (WHO), it has been shown that approximately 50 million people are suffering from dementia, of which 60 -70% are diagnosed with Alzheimer's Disease, and it is estimated that the case will double in the next ten years.

Taking into consideration that AD-specific pathology begins decades before the onset of dementia, suggesting that this pathology may be influenced by inflammatory conditions present early in life. Periodontitis, in its chronic or aggressive form, can affect the young population.

With the increasing rate of PD, it was enthralling to explore whether there is a high rise among young subjects seeking treatment at the Prosthodontics Department, University of Medicine and Pharmacy “Victor Babes” Timisoara, Faculty of Dental Medicine.

The following objectives were pursued in this Ph.D. thesis:

1. Screening young subjects from 18-45 years of age to explore the occurrence of aggressive periodontitis (AgP), based on examination of panoramic radiographs and association with oral environmental factors, and characterize the prevalence of these patients in the western region of Romania, Europe.
2. To determine if people with markers of periodontal disease (clinical, inflammatory, radiographical) have changes in cognition and cognitive decline. This association was explored by further comparing the results from salivary inflammatory cytokines and psychological memory tests of people with and without periodontal disease.
3. Exploring salivary metabolic signature among the subjects, with and without PD, in our young convenience sample group

The present PhD thesis is structured in two parts:

The **GENERAL PART** consists of four chapters.

The first two chapters describe the current stage of knowledge with regard to PD and AD, bringing the theoretical arguments which serve as cornerstones for the doctoral thesis and include recent literature. Each disease is distinctively described along with the diagnostical aspects, classification, etiology, and risk factors.

The 3rd chapter emphasizes the connection between PD and AD. In addition, two peripheral factors that are involved and have a significant role are inflammatory and infectious mechanism. Two neurological proteins that have a defective function and disrupt the activity of neurons by eliminating toxic substances are associated with the onset of BA. Fragments of beta-amyloid protein accumulate by participating in the creation of amyloid plaques and interfering with intraneuronal communication. On the other hand, the proteins that support the transport of nutrients for neurons change their shape and cause "tangles" of the neural cell network.

The inflammatory hypothesis is commonly accepted to be a significant etiologic or contributory factor in which there is a reaction between senile plaques with antibodies in contrast to the glial cell production of proinflammatory cytokine (TNF- α , IL-1 β , IL-6) and C-reactive protein (CRP). When taking into consideration the infectious hypothesis, microbiota can be the result of a disrupted homeostatic balance and a predictor of immune system decline. In addition, it can affect antimicrobial resistance and stimulate further bacterial colonization. In addition, the autoimmune mechanism is present both in healthy as well as individuals with PD, but in the second, more vulnerable group, it can result in tissue damage and alteration on the systemic level.

The 4th chapter highlights relevant biomarkers in AD and describes the importance in using saliva as non-invasive biological fluid. Moreover, it can serve as a potential biomarker for the screening of amyloidosis (A β 42), P-tau, T-tau, Lactoferrin, Acetylcholinesterase, oral microbiome and metabolites, oxidative stress markers, salivary flow, antioxidants and oxidative damage products and genetic biomarkers

The **SPECIFIC PART** consists of **3 studies** and serves as an overview of the research conducted by the Ph.D. student and includes INTRODUCTION, MATERIAL AND METHOD, RESULTS, DISCUSSIONS and CONCLUSIONS for each study in part.

Since the long-term goal of this study is to investigate the contribution of these inflammatory conditions to AD pathology, the first step was to explore the occurrence of aggressive periodontitis in our population and characterize it. While it is not known what the decisive factors in the pathogenesis of Alzheimer's disease are, it is assumed that inflammation and infection are involved, the nature and mechanism of these relationships are currently being studied

Another essential factor for this research is establishing whether oral inflammation and dysbiosis could predict Alzheimer's Disease Development.

MATERIAL AND METHODS

The first study direction was focused on screening young convenience samples for PD prevalence. The study population consisted of patients presenting consecutively to the Prosthodontics Department of the Faculty of Dental Medicine, "Victor Babeş" University of Medicine and Pharmacy, Timisoara. These subjects were enrolled in the period from 2013 to 2016, consented to our study, and fulfilled our research criteria. The study design was approved by the University Ethics Committee.

In general, patients seeking prosthodontic treatment were referrals from other school departments (approximately 80%) or were self-referred. Annually, approximately 800–900 patients of all ages are seen at the Prosthodontics Department. Subjects were included in the study if they were age ≤ 42 and were not edentulous. Subjects were excluded if they had a history of uncontrolled hypertension, diabetes, radiation, and/or drug use.

The diagnosis and classification of the periodontal conditions, as well as dental pathologies and conditions, were based on panoramic radiographs. The radiographs were visually evaluated by two calibrated periodontists (AK, RG) and examiners (AK, SH). The radiographs were rated as optimal quality since the information provided was sufficient to obtain diagnostic information.

The second study focus was on neuropsychological screening for a group of subjects from the previous sample and a biological sample collection for pro-inflammatory cytokine tests.

This is a cross-sectional comparative study of 3 clinical groups of young, medically healthy subjects from the western region of Romania. The subjects were recruited from a pool of 149 subjects that participated in a previous study . These subjects presented to the Prosthodontics Department of the Faculty of Dental Medicine, Victor Babeş University of Medicine and Pharmacy, Timisoara, for comprehensive dental treatment.

This study was approved by the University Ethics Committee. Informed consent was reviewed and signed with all subjects. (No27/2017). Forty subjects were recruited: 10 with aggressive periodontitis (AGG_P), 20 with chronic mild-moderate periodontitis CrP, and 10 with no signs of periodontitis NL. In addition to fulfilling the inclusion and exclusion criteria described below, subjects were required to agree to a neuropsychological evaluation and saliva collection. Diagnosis of periodontal conditions was done by two calibrated periodontists using panoramic radiographic as we previously published. Radiographic images were also used to assess caries, tooth number, endodontic treatments, and periapical pathology.

AD-specific pathology begins decades before the onset of dementia, suggesting that this pathology may be influenced by inflammatory conditions present early in life. Periodontitis, in its chronic or aggressive form, can affect a young population.

Neuropsychological assessments were performed by a clinical psychologist using RAVLT, MOCA, MMSE, and Prague tests. Romanian translations of each test were used.

The primary outcome measure was delayed recall tested with the Rey Auditory Verbal Learning Test (RAVLT). Secondary outcomes were immediate memory and learning assessed by Rey Auditory Verbal Learning Test (RAVLT). In addition, the Montreal Cognitive Assessment test (MOCA), Mini Mental State Examination (MMSE) and Prague tests were also used.

Neuropsychological assessment is paramount in establishing mild cognitive dysfunction and subsequently keeping the patient under observation to follow the evolution of the disease. The evaluation of the cognitive functions consisted of examining the short and long-term memory and the concentration capacity (PRAGA test). Also, the orientation abilities, praxis, language, and executive functions were evaluated for these aspects, using the following standardized tests: MMSE, MOCA, REY (Delayed recall test), Clinical Dementia Rating Scale, FAQ (Functional

Activities Questionnaire), and CGI (Global Clinical Impression). Because depression can mimic the signs of cognitive dysfunction, in advanced cases, even dementia, the Hamilton test for dementia was applied (short variant - 17 items), the presence of depression being one of the exclusion criteria in this study.

Saliva collection and processing were done as published. Salivary stimulation was achieved by chewing unflavored chew paraffin wax pellets (Gleegum, Verve Inc., Providence, RI). Saliva was stored at -80C until cytokine assays were performed. Salivary Interleukin-1 (IL-1 β) and tumor necrosis factor- α (TNF- α) levels were assessed using human IL-1 β ELISA kit (Invitrogen, Thermo Fisher Scientific, CA, USA) and Human TNF- α Ultrasensitive ELISA kit (Invitrogen, Thermo Fisher Scientific, CA, USA) using the manufacturer's protocol. The absorbance was read spectrophotometrically at 450 nm using a GloMax Discover instrument v3.0. (Promega Corp, WI, USA). Using the standard equation curves, saliva IL-1 β and TNF- α concentrations were determined.

The third study extends the avenues of connections between PD and AD on a metabolomic level by focusing on relevant periodontal metabolites levels that show distinctive concentrations among the three study groups and express their unique pathways as a keystone in cognitive decline.

A total of 40 subjects (ages between 18 and 43) were enrolled in this study at the Prosthodontics Department from the Faculty of Dental Medicine, "Victor Babeş" University of Medicine and Pharmacy, Timișoara, Romania. From the total number of participants, 10 presented AgP, 20 had CrP (10 with CrP mild and 10 with CrP) and 10 were healthy volunteers (NL) used as controls, each with at least six existent teeth. They were matched by age, demographics, etiology of losing teeth, family history, education, clinical and X-ray criteria. All subjects were diagnosed using a modified published diagnostic criterion.

Subjects selected for saliva collection did not undergo any dental cleaning procedure nor used antibiotics for at least six months prior to sample collection. They had no alcohol intake for at least 12 hours and were not to be eating or smoking for at least two hours before of their saliva collection.

The collection tube is placed into a cup containing ice for 10 min before saliva collection begins. The collection tube remains in the ice filled cup for the entire collection period. The

subject is given unflavored gum base and told to chew and NOT to swallow for as long as is needed to collect 5 ml or until the subject is tired. The subject gently spits saliva into the tube. The recording volume along with start and stop times are establishing the salivary flow rate. The collection is stopped at 5ml and may take about 15 minutes.

Management of saliva sample; after sample collection, saliva is submitted to the laboratory for processing. The sample is taken within 1 hour to the bio safety hood and 2.5 ml Whole saliva is aliquoting into 500 µl tubes and store at -80C until use. The sample is spun at 2,500 rpm for 25 min at 4 °C (Fisher Brand AccusSpin 1R centrifuge with 4 place swinging bucket rotor). After the rotor stops, the buckets are removed from the centrifuge with covers in place and opened in the biosafety hood. In the hood, the tubes are removed from the bucket and the supernatants carefully decanted, while not disturbing the Pellet, then placed into a new tube and gently mixed. The saliva is aliquoted into 250µl aliquots, labeled, placed into a labeled freezer box, and stored at -80 °C. This saliva is the centrifuged saliva used for cytokines, proteomics and metabolomics.

RESULTS AND DISCUSSIONS:

The findings in this extended research raise the possibility that in young subjects with periodontal disease abnormal memory dysfunction is present, signs of brain abnormalities may exist, and increased risk of AD later in life is possible.

The first study showed that the prevalence of periodontal disease in young subjects presenting to a prosthodontics department at a university in Western Romania was high. Among the 149 patients seeking prosthodontic rehabilitation, only 34.2 % were periodontal disease free while 65.8% had periodontal disease. Among those with periodontal disease, 82.7% had radiographic diagnosed chronic periodontitis and 17.3% had aggressive periodontitis. Our proposed hypothesis of low prevalence of periodontal disease was not supported. Our results suggest that very few people, or none at all, received periodontal treatment prior to seeking prosthodontic rehabilitation. Indeed, in our study, only six of 76 subjects reported having scaling and root planning prior to coming to the Prosthodontics department and 40% professional cleaning.

Our study has several limitations, among them, the importance of limited applicability of these results to the younger population of western Romania. This study included only the younger subjects coming to the Prosthodontics department while excluding those with diabetes or other significant medical conditions. The included subjects were less than 42 years of age, but the majority of the subjects were older than 30. Other limitations are related to classification and selection bias.

In the second study, the findings raise the possibility that in young subjects with periodontal disease abnormal memory dysfunction is present, signs of brain abnormalities may exist, and increased risk of AD later in life is possible.

Episodic the episodic memory is thought to be the first memory domain to be impaired in AD. Studies showed that in addition to delayed recall, learning curves are also impaired in those with MCI compared to those with normal cognition. These tests are early predictors of AD and can differentiate between cognitively normal and patients with MCI. Impairments in these cognitive tests have been associated with brain neurodegeneration and the lesions of AD. Immediate recall also depends on the learning ability and information coding and these impairments have been associated with atrophy in frontal as well as temporal lobe while delayed recall task was associated with the medial temporal area. Early memory impairment is found to associate with early AD with pathological findings localized in the mesial temporal lobes, especially in the hippocampal formation and entorhinal cortices .

The difference in cognitive tests between NL and those with AgP is consistent across multiple cognitive tests. These results are not surprising as AgP is highly destructive and associates with more severe immune response compared to CrP. The microbial load is also higher and characterized by many pathogenic bacteria. The difference between those with CrP and NL is not as consistent. This may be due to less severe periodontal disease, less aggressive immune response or less microbial burden. An additional reason could be the limited sample size. The cognitive tests for CrP were slightly lower than those of NL and therefore a larger number could result in significance.

The third study's aim was to investigate possible differences in metabolites levels in patients with different stages of periodontitis (CrP mild, CrP and AgP) relative to healthy controls

(NP), and in between the groups of patients compared to the aggressive phenotype. To the best of our knowledge, there is no available literature data that assesses inter-group comparisons from periodontitis patients using chip-based high-resolution mass spectrometry (QTOF MS). We found a generally altered metabolism of patients with periodontitis compared to NP, with majority of metabolites having significantly higher levels ($p < 0.05$) relative to controls, in all stages of the periodontal disease. In addition, findings indicated a general trend of metabolites levels from patients suffering from AgP to be significantly elevated compared to the chronic phenotypes. Of interest, cadaverine, tyrosine and valine/5-aminovaleric acid/betaine were significantly increased in all stages of periodontal disease.

The emerging field of metabolomics has proven to possess a promising potential of discovering novel metabolites in various biological samples, that could eventually serve as candidate biomarkers in a vast number of conditions, such as cancer (oral cancer included), neurodegenerative diseases (i.e. Alzheimer's disease), and periodontal disease. These biomarkers could enrich the common diagnostic procedures used in present, by granting new opportunities of early diagnostic, with higher specificity, sensitivity and less invasiveness. In addition, metabolic profiling could become useful in discriminating the stages of periodontitis based on the specific metabolomic signature of biomarkers.

Nevertheless, salivary metabolomics is the only approach capable of providing an end- point snapshot of the dynamic metabolic pathways involved in the pathogenesis of periodontitis, thus being a valuable source of key information regarding its complex biochemistry.

CONCLUSIONS:

The first study showed that young people coming to the Prosthodontics department seeking oral rehabilitation have a high prevalence of periodontal disease. Close collaboration between different dental specialties in a university setting is required.

The second study showed that young subjects with periodontal disease had lower cognition. There is a need for more observational studies on this topic with control of modifiable variables (diagnostic criteria, time of diagnosis and follow-up between periodontitis and cognitive decline, level of education, etc.) to investigate the cause-effect relationship between the pathologies. Continued investigation of modifiable variables in AD, such as periodontal disease,

provides new directions for treatments and therapies which could considerably alter the future impact of AD.

The third study identified distinctive metabolic signatures associated with inflammation and dysbiosis, changes that can influence the way we look at the prevention, treatment and diagnostic biomarkers of periodontal disease. These results need further validation in large-scale powered studies in order to confirm our findings and retrieve new insights into the biology of periodontitis.

This multidirectional research managed to detect an unequivocal biochemical signature in discriminating CRP from AgP. It has been observed that the aggressive phenotype reveals a distinct metabolic signature. In addition, neuropsychological tests had shown that cognitive decline is more present in the AgP group than NL or CRP. This dynamic portrait of generally altered metabolism and proper neurological/neuropsychological exam can aid in the early detection of mild cognitive impairment.

In this contemporary era of digital dentistry, it is paramount to have a periodontally healthy patient for prosthodontic treatment, especially when planning for a long-term esthetic and functional outcome. Identifying modifiable risk factors, together with monitoring and treatment of PD, could reduce or postpone the AD diagnosis.

The potential long-term impact of this project is substantial towards understanding the early contributors to AD-pathology in the brain. Brain atrophy and cognitive decline may ultimately lead to the identification of additional AD biological markers, such as prevention, early diagnostic, and treatment protocols for AD.