UNIVERSITY OF MEDICINE AND PHARMACY

„CAROL DAVILA", BUCHAREST

DOCTORAL SCHOOL

FIELD: MEDICINE

BIOCHEMICAL ASSESSMENT IN DENTAL PULP PATHOLOGY

SUMMARY OF PhD THESIS

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2022

Contents

Abbreviation List

BD – Beta-defensins

BMP – Bone morphogenetic protein

CNC – Cranial neural crest

CNS – Central nervous system

CGRP – Calcitonin-gene related peptide

DC – Dendritic cells

DMP-1 – Dentine matrix protein-1

DPP – Dentine phosphoprotein

DSP – Dentine sialoprotein

ECM – Extracellular matrix

EDJ – Enamel-dentine junction

ELISA – Enzyme-linked immunosorbent assay

FGF-2 – Fibroblast growth factor-2

FNE – Free nerve ending

GM-CSF – Granulocyte macrophage-colony stimulating factor

GP – Glycoproteins

IFN-γ – Interferon gamma

IL – Interleukin

iTreg – induced regulatory T cells

IQR – Interquartile range

LPS – Lypopolysaccharides

LTA – Lipoteichoic acid

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinases

MPO – Myeloperoxidase

MSC – Mesenchymal stem cells

NGF – Nerve growth factor

NK – Natural Killer cells

NPY– Neuropeptide Y

NSB wells – Non-specific binding wells

PAMP – Pathogen-associated molecular pattern

PBS – Phosphate-buffered saline

PDGF – Platelet-derived growth factor

PG – Proteoglycans

PMN – Polymorphonuclear neutrophils

PT – Permanent teeth

QC wells – Quality control wells

RANKL – Receptor activator of nuclear factor-ϰB ligand

ROS – Reactive oxygen species

SD – Standard deviation

SOD – Superoxide dismutaze

SP – Substance P

TGF-β1 – Transformig growth factor-β1

Th – T helper cells

TLR – Toll-like receptor

TMB - Tetrametylbenzidine

TNF-α – Tumour necrosis factor alpha

Treg – Regulatory T cells

Ts – Suppressor T cells

TT – Temporary teeth

VEGF – Vascular endothelial growth factor

VIP – Vasoactive intestinal polypeptide

WHO – World Health Organization

INTRODUCTION

Dental pulp inflammation (pulpitis), both in primary and permanent dentition, takes place in 2 stages, reversible and irreversible. Each of these stages is followed by a different dental treatment. In the case of reversible pulpitis, the preservation of the vital dental pulp tissue is essential especially in young permanent teeth, while irreversible pulp dental pulp inflammation requires complete removal of infected tissue, disinfection, and filling of the endodontic space [1]. Often the forms of irreversible inflammation predominate over the reversible ones, requiring more complex treatments.

The installation of pulpal inflammation caused by bacterial invasion is accompanied by the release of various mediators, many of them having synergistic or antagonistic role, causing changes dental pulps' molecular and cellular levels [3]. The literature is characterized by lack of studies based on molecular determinations in dental pulp tissues obtained from children and adolescents.

The clinical diagnosis of pulpal pathology is usually made in the late stages of inflammation and also may be inaccurate and subjective [5]. The development of procedures and tools for accurate diagnosis of dental pulp pathology and minimally invasive approaches are essential especially for the differential diagnosis and treatment of reversible and irreversible inflammation of temporary (primary) and permanent teeth.

The aim of this research was to identify the presence and concentration of various mediators (TNF-α, IL-2, IL-17, SOD-3, Catalase, MMP-7, MMP-9, Osteocalcin and TGF-β1) that participate in the inflammatory processes of temporary and permanent teeth and to understand the mechanisms of pulpal inflammation which can be helpful in the establishment of protocols for accurate diagnosis that will be followed by the most appropriate treatment methods.

I. GENERAL PART

The general part includes 3 chapters with relevant information on the topic addressed in the personal contributions part, as well as a descriptive analysis of past achievements in the field, based on the literature in Romania and abroad.

In the **first** chapter a detailed description of the dental pulpal tissue was made in terms of its origin, structure, vascularization, innervation, and functions.

Chapter 2 provides information on bacterial dental pulp inflammation, the role of odontoblasts in the defense of dental pulp and the innate and acquired immune response of the pulp tissue. It also describes the changes that occur macroscopically in the dental pulp, aspects regarding the symptoms and clinical examination in pulpitis cases, as well as the mechanisms of reparation and regeneration of the pulpo-dentinal complex.

Chapter 3 includes general information about biomarkers and their use in various local and systemic pathologies. In addition, a detailed description of numerous previous studies assessing the presence of biomarkers in conditions of dental pulp inflammation has taken place.

II. PERSONAL CONTRIBUTIONS

Chapter 4. General hypothesis and objectives

The hypothesis of this research was that the existence of biochemical changes in the dental pulp can be determined using biomarkers. To test this hypothesis, irreversibly inflamed and healthy dental pulp samples were collected from temporary (TT) and permanent teeth (PT).

Therefore, this research is based on 3 studies that have the following general objectives:

1. Assessment of the concentration of biomarkers of inflammation, oxidative stress, extracellular matrix (ECM) degradation and reparation and mineralization mechanisms in inflamed dental pulp compared to dental pulp samples obtained from healthy teeth, both temporary and permanent.
2. Establishment of correlations between different types of biomarkers in the primary and permanent dentition.
3. Comparison of the concentration of biomarkers in acute inflamed pulp tissues from temporary teeth *versus* permanent teeth.

Chapter 5. General methodology

Patients' selection

All 3 studies included children and adolescents aged 6-18 years old who sought treatment in the Pedodontics Department of "Carol Davila" University of Medicine and Pharmacy, Bucharest.

General criteria for patients' inclusion: patients without general (diabetes, metabolic diseases etc.) or local conditions (aphthous ulcers, herpetic stomatitis, sores, wounds), without general/local medication (in the last 24 hours), parents have signed the informed consent, cooperative patients, patients that accept local anaesthesia and isolation using rubber dam.

General criteria for patients' exclusion: general/local conditions, general / local medication, uncooperative patients, patients who do not accept local anaesthesia and/or use of rubber dam isolation.

Dental pulp tissue collection

Following the establishment of the clinical diagnosis by corroborating data from patient's history, clinical examination, examination of radiographs and information obtained from paraclinical tests, the appropriate treatment was selected, being represented by extractions or vital pulpectomies. All treatments were realized under aseptic conditions (professional cleaning, cleaning of the tooth and mucosa with antiseptic solutions in order to perform anaesthesia). Local anaesthesia was given by infiltration or topical application (for temporary teeth with mobility).

The study group in all studies was represented by samples of irreversibly inflamed pulp tissue (TT/PT) obtained by vital pulpectomy while the control group consisted of samples of healthy pulp tissue (TT/PT) collected after extraction and tooth sectioning. A single sample of dental pulp tissue was collected from each patient.

Dental pulp samples were frozen at -70 ^{o}C until further processing for obtaining the pulp lysate. Dental pulp samples processing and ELISA determination took place in the Biochemistry Department of "Carol Davila" University of Medicine and Pharmacy, Bucharest.

Pulp lysates preparation

All frozen pulp samples were thawed at room temperature. PBS solution was added to each container with sample and was crushed using a glass rod. Subsequently, the samples were sonicated for 3 minutes, followed by centrifugation for 10 minutes at 4000 rpm to obtain the supernatant.

Assessment of the level of biomarkers using ELISA method

The levels of IL-2, IL-17, MMP-7, MMP-9, TNF-α, SOD3, Catalase, Osteocalcin and TGF-β1 were determined by the ELISA sandwich method according to widely known methods [7] using commercial kits from Elabscience (Houston, TX, USA) and a semi-automated ELISA STAT FAX 303-PLUS analyzer from Awareness Technologies (Palm City, FL, USA). The general protocol of the sandwich ELISA method is summarized in Figure 5.1.

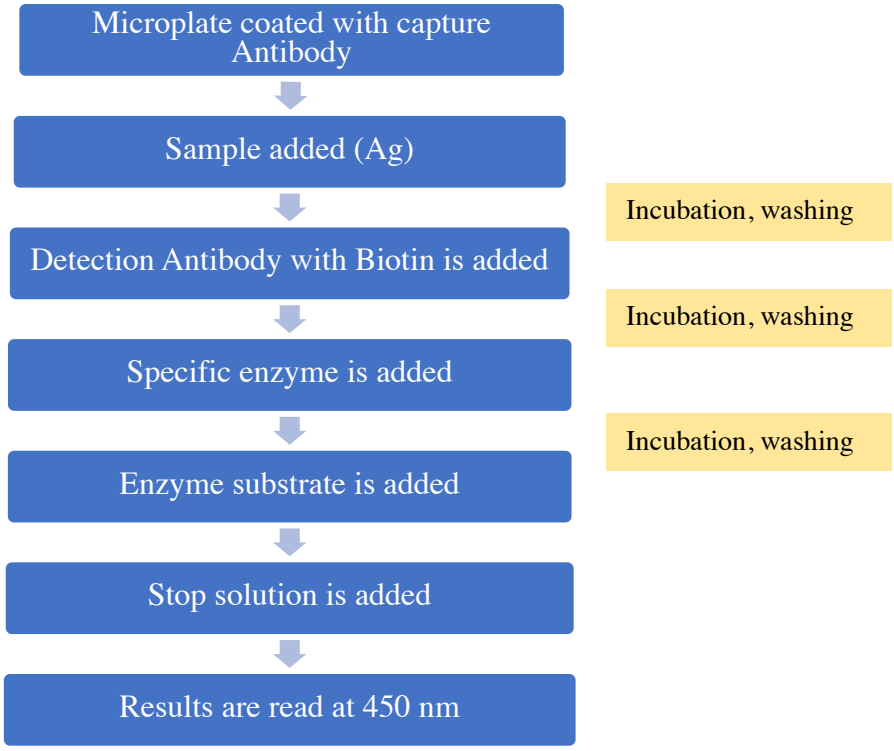


Figure 5.1. General protocol for the sandwich ELISA method

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 25 and Microsoft Office Excel / Word 2013. Quantitative variables were tested for distribution using the Shapiro-Wilk test and were expressed as averages with standard deviations (SD) or medians with interpercentile intervals (IQR). Qualitative variables were expressed as absolute or as a percentage.

Non-parametric independent quantitative variables were tested using the Mann-Whitney U test. Parametric independent quantitative variables were tested using the Student/Welch test. These statistical tests were applied in all 3 studies. A value of $p \leq 0.05$ was considered statistically significant.

General clinical and laboratory protocol

- Brushing, scaling, cleaning of teeth and mucosa with alcohol.
- Local infiltration anaesthesia for vital pulpectomies and extractions of PT and TT/ just topical anaesthesia for TT extraction with increased mobility.
- Rubber dam isolation before vital pulpectomies.
- Extraction and sectioning of PT/TT for the control group/Removal of decayed enamel and dentine and access to the endodontic space of PT/TT for the study group.
- Gentle collection of dental pulp with new, sterile instruments.
- Placement of dental pulp samples in sterile tubes with saline solution and frozen.
- Processing of samples to obtain pulp lysates.
- Determination of biomarkers concentration using the ELISA sandwich method

Chapter 6. Biochemical assessment in dental pulp samples from permanent teeth

6.1. Introduction

- The **aim of the study** was to identify differences in the levels of TNF-α, IL-2, IL-17, SOD-3, Catalase, MMP-7, MMP-9, Osteocalcin and TGF-β1 in acute inflamed pulp samples compared to healthy pulp tissues from PT.

- The **objectives of the study** were to determine the concentration of biomarkers of inflammation (TNF-α, IL-2, IL-17), oxidative stress (SOD-3, Catalase), ECM degradation (MMP-7, MMP-9) and reparation and mineralization (Osteocalcin, TGF-β1) in acutely inflamed pulp tissues compared to healthy pulp samples from PT.

6.2. Material and method

The study included 42 patients aged 6-18 years old. Criteria for patient's inclusion in the study are described in the general methodology chapter.

- *Inclusion criteria for teeth:* PT with symptomatic irreversibly inflamed pulp tissue (study group), healthy, free of caries, without associated trauma or periodontal disease PT (control group).
- *Exclusion criteria for teeth:* PT with partial/asymptomatic inflammation/necrosis, PT without symptoms, but with the presence of carious lesions, trauma, periodontal disease, PT with internal / external resorptions, endodontic treatments, calcifications.
- The **study group** consisted of samples of symptomatic irreversibly inflamed pulp tissues obtained by vital pulpectomy from PT with clinical diagnosis of acute pulpitis (n=23) that required emergency treatment.
- The **control group** was represented by healthy pulp tissues collected from healthy PT (n=19) extracted for orthodontic reasons [33].

General clinical and laboratory protocol is described in the "General methodology" chapter. Statistical analysis of the experimental results was performed using the Shapiro-Wilk, Mann-Whitney U, Student/ Welch tests depending on the type and distribution of each variable.

6.3. Results

Mean age ± standard deviation (DS)

- Total group (n=42): 10.98 ± 3.65 years old
- Patients in the control group (n=19): 10.91 ± 3.18 years old
- Patients in the study group (n=23): 11.06 ± 4.25 years old.

All biomarkers were detected in both the study and control groups. Average values and statistical differences between the 2 groups are shown in Table VI.1.

Table VI. 1. Levels of IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin and TGF-β1 in acute irreversibly inflamed *versus* healthy pulp tissues from PT

Biomarker	Control group (n=19), Mean value ± SD	Study group (n=23), Mean value ± SD	Statistical significance (p)
IL-2 (pg/mg prot.)	22.23 ± 4.66	113.21 ± 29.14	**<0.001***
IL-17 (pg/mg prot.)	18.2 ± 2.04	138.04 ± 16.54	**<0.001***
TNF-α (pg/mg prot.)	6.342 ± 3.429	54.435 ± 11.195	**<0.001***
MMP-7 (ng/mg prot.)	1.853 ± 0.59	2.3 ± 0.85	0.061**
MMP-9 (ng/mg prot.)	1.568 ± 0.44	1.496 ± 0.5	0.625**
SOD-3 (ng/mg prot.)	2.142 ± 0.97	26.32 ± 7.71	**<0.001***
Catalase (U/mg prot.)	2.258 ± 0.66	2.67 ± 0.74	0.064**
Osteocalcin (ng/mg prot.)	2.258 ± 0.66	7.97 ± 4	**<0.001***
TGF-β1 (pg/mg prot.)	12.12 ± 2.54	23.16 ± 11.79	**<0.001****

(prot.=protein, * Welch T-Test, ** Student T-Test, *** Mann-Whitney U Test)

6.4. Discussions

IL-2, IL-17, TNF-α, SOD-3, Osteocalcin and TGF-β1 showed increased concentrations in irreversibly inflamed pulp tissue samples compared to healthy pulp samples, the differences between the 2 groups being significant ($p \leq 0.05$). These results are consistent with most of the results of previous studies although they have used pulp tissue samples from adult patients [76] [101] [142] [151] [152] [153] [155] [156] [158] [159] [164,165].

Regarding the IL-2 levels, Anderson et al. [143] and ElSalhy et al. [76] reported absence of statistically significant differences ($p>0.05$) between irreversibly inflamed and healthy pulp tissues.

Tulunoglu et al. [8] did not report any significant differences of the total SOD activity in irreversibly inflamed pulp tissue samples *versus* healthy dental pulp tissues collected from children's PT while Varvara et al. [160] observed a significantly lower activity ($p<0.001$) of Cu, Zn-SOD in irreversible dental pulp inflammation compared to healthy dental pulp samples. The results of the mentioned studies may be due to the progression of inflammation and destruction of the enzyme.

In our study the enzymes MMP-7, MMP-9, and Catalase did not show significant differences between study and control group ($p>0.05$), the results being in contradiction with other studies that reported significantly increased concentrations in pulpal inflammation

[7,103,108,112] [169]. The differences between the results of this study and previous researches may be due to the molecular diagnostic methods used, the different target (proteins, enzymatic activity) or variations in the intensity of stimuli that cause the production of these enzymes, even when similar clinical symptoms are present.

No studies have been identified assessing the presence of MMP-7 in dental pulp tissues. MMP-7 proteins and mRNA were determined in inflammatory tissue lesions collected from chronic apical abscesses and healthy periodontal ligament samples, with significantly increased results in the pathological samples [171].

In irreversible dental pulp inflammation, the treatment aims to completely remove of the infected pulpal tissue, sterilization, and filling of the endodontic space. PT with such treatments may become more fragile, with higher risk of fracture. In the case of young PT with open apex, removal of the inflamed dental pulp should be followed by the initiation of apexification procedure in order to form a hard apical barrier in the absence of growth of the root. Root walls remain thick and short having also high risk of fracture.

Molecular procedures can be useful in accurate establishment of dental pulp diagnosis ideally as early as possible, in reversible stages which will be followed by vital, less invasive treatments, especially in the case of immature PT leading to apexogenesis.

Chapter 7. Biochemical assessment in dental pulp samples from temporary teeth

7.1. Introduction

The aim of the study was to evaluate the levels of IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin and TGF-β1 in acute pulpitis, chronic pulpitis and healthy dental pulp samples obtained from TT.

Specific Objective #1: Determination of the concentration of IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin and TGF-β1 in acutely inflamed pulp tissues *versus* healthy pulp tissues collected from TT.

Specific Objective #2: To assess the levels of IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin, and TGF-β1 in chronically inflamed *versus* healthy TT pulp samples.

Specific Objective #3: Comparative assessment of IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin and TGF-β1 levels in acute and chronic TT pulpitis.

7.2. Material and method

This study included 46 subjects aged 6-12 years old. A single pulp tissue from TT was collected from each patient according to the inclusion criteria. Patient's inclusion and exclusion criteria are described in the "General methodology" chapter.

Inclusion criteria for teeth: TT with irreversible symptomatic inflammation (acute total pulpitis), TT with irreversible asymptomatic inflammation (closed chronic pulpitis), TT that required extraction due to increased mobility (physiological root resorption)/ TT with persistent retention on the dental arch.

Exclusion criteria for teeth: TT with acute partial pulpitis, TT with chronic partial/open pulpitis, TT with necrosis, TT with perforations / pathological resorptions (internal / external), TT with acute/ chronic periapical lesions.

Study group 1 was represented by acutely inflamed dental pulp tissues (n=18) obtained by vital pulpectomy from TT with clinical diagnosis of acute (symptomatic) irreversible pulpitis. Patients from whom these samples were collected required emergency treatment.

Study group 2 consisted of chronically inflamed dental pulp tissues (n=15) collected by vital pulpectomy from TT with clinical diagnosis of chronic (asymptomatic) closed pulpitis. The patients from whom these samples were collected sought treatment for what they presumed to be simple caries, but after objective examination proved to have extensive pulp pathology.

The control group included samples of healthy dental pulp tissues (n=13) obtained from clinically healthy TT with increased mobility due to physiological root resorption or TT with prolonged retention on the dental arch that required extraction.

General clinical and laboratory protocol is described in the "General methodology" chapter. Statistical analysis of the experimental results was performed using the Shapiro-Wilk, Mann-Whitney U, Student / Welch tests depending on the type and distribution of each variable.

7.3. Results

Mean age ± SD of all patients (*n*=46): 8.58 ± 1.39 years old. Control group consisted of 13 samples of healthy pulp tissues obtained from 13 patients 8.35 ± 1.46 years old. The acute pulpitis group was obtained from 18 patients 8.8 ± 1.06 years old, while samples with chronic closed inflammation were collected from 15 patients 8.52 ± 1.68 years old.

Proteins of all biomarkers were detected by ELISA method in all dental pulp samples that were analyzed (Table VII.28.-VII.30.).

Table VII. 28. IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin, and TGF-β1 levels in irreversibly acute inflamed *versus* healthy pulp tissues from TT

Biomarker	Control group (*n*=13), Mean value ± SD	Acutely inflamed group (*n*=18), Mean value ± SD	Statistical significance (*p*)
IL-2 (pg/mg prot.)	21.44 ± 5	108 ± 32.77	**<0.001***
IL-17 (pg/mg prot.)	18.14 ± 2.6	130.71 ± 12.38	**<0.001***
TNF-α (pg/mg prot.)	6.75 ± 3.15	57.71 ± 10.78	**<0.001*****
MMP-7 (ng/mg prot.)	1.63 ± 0.78	2.25 ± 0.69	0.071***
MMP-9 (ng/mg prot.)	1.59 ± 0.32	1.44 ± 0.53	0.464**
SOD-3 (ng/mg prot.)	1.56 ± 0.44	27.45 ± 8.31	**<0.001***
Catalase (U/mg prot.)	2.35 ± 0.6	2.59 ± 0.75	0.187**
Osteocalcin (ng/mg prot.)	2.51 ± 1	5.87 ± 3	0.002*
TGF-β1 (pg/mg prot.)	13.24 ± 1.48	18.07 ± 4.5	**<0.001***

Table VII. 29. IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin, and TGF-β1 levels in chronically inflamed *versus* healthy pulp tissues from TT

Biomarker	Control group (*n*=13), Mean value ± SD	Chronically closed inflamed group (*n*=15), Mean value ± SD	Statistical significance (*p*)
IL-2 (pg/mg prot.)	21.44 ± 5	121.33 ± 21.67	**<0.001***
IL-17 (pg/mg prot.)	18.14 ± 2.6	149.44 ± 16.21	**<0.001***
TNF-α (pg/mg prot.)	6.75 ± 3.15	49.33 ± 10.38	**<0.001***
MMP-7 (ng/mg prot.)	1.63 ± 0.78	2.36 ± 1.1	0.120**
MMP-9 (ng/mg prot.)	1.59 ± 0.32	1.57 ± 0.47	0.960**
SOD-3 (ng/mg prot.)	1.56 ± 0.44	26.29 ± 7.16	**<0.001***
Catalase (U/mg prot.)	2.35 ± 0.6	2.81 ± 0.75	0.060**
Osteocalcin (ng/mg prot.)	2.51 ± 1	11.23 ± 3.13	**<0.001***
TGF-β1 (pg/mg prot.)	13.24 ± 1.48	31.06 ± 15.3	**0.006***

Table VII. 30. IL-2, IL-17, TNF-α, MMP-7, MMP-9, SOD-3, Catalase, Osteocalcin, and TGF-β1 levels in acutely *versus* chronically closed inflamed pulp tissues from TT

Biomarker	Acutely inflamed group (*n*=18), Mean value ± SD	Chronically closed inflamed group (*n*=15), Mean value ± SD	Statistical significance (*p*)
IL-2 (pg/mg prot.)	108 ± 32.77	121.33 ± 21.67	0.295**
IL-17 (pg/mg prot.)	130.71 ± 12.38	149.44 ± 16.21	**0.005****
TNF-α (pg/mg prot.)	57.71 ± 10.78	49.33 ± 10.38	0.159***
MMP-7 (ng/mg prot.)	2.25 ± 0.69	2.36 ± 1.1	0.926***
MMP-9 (ng/mg prot.)	1.44 ± 0.53	1.57 ± 0.47	0.543**
SOD-3 (ng/mg prot.)	27.45 ± 8.31	26.29 ± 7.16	0.986**
Catalase (U/mg prot.)	2.59 ± 0.75	2.81 ± 0.75	0.506**
Osteocalcin (ng/mg prot.)	5.87 ± 3	11.23 ± 3.13	**0.001****
TGF-β1 (pg/mg prot.)	18.07 ± 4.5	31.06 ± 15.3	**0.035***

(prot.=protein, * Welch T-Test, ** Student T-Test, *** Mann-Whitney U Test)

7.4. Discussions

Some of the studied biomarkers were previously included in other research but their concentrations were evaluated in dental pulp samples obtained from PT, most of them from adult patients. In consequence, the results of the present study were compared with studies that determined the levels of these molecules in inflamed dental pulp tissues collected from PT.

TNF-α, IL-2, IL-17, SOD-3, Osteocalcin and TGF-β1 showed higher levels in the study groups compared to the control group of TT, the differences being statistically significant ($p \leq 0.05$). The results of the present study are similar to those of Pezelj-Ribaric et al. [155], Kokkas et al. [153] and Abd-Elmeguid et al. [101], who used ELISA, RT-PCR, Multiplex as diagnostic tools and demonstrated significant increase in TNF-α concentrations in acutely inflamed dental pulp tissues compared to healthy samples. Rauschenberger et al. [142] reported significantly increased protein concentrations of IL-2 in symptomatically inflamed pulp tissues in contrast to Anderson et al. [143] who did not find any significant differences between symptomatic pulpitis and healthy teeth.

Liu et al. [152] and Xiong et al. [151] determined the presence of IL-17 in symptomatic irreversibly inflamed dental pulp samples with significant differences compared to healthy teeth. Regarding the levels of SOD-3, our findings are similar to those of Bodor et al. [156]

and Davis et al. [158] and in contrast to Tulunoglu et al. [8] and Varvara et al. [160] who observed a significant increase in SOD synthesis in healthy teeth *versus* those with pulp inflammation.

Abd-Elmeguid et al. [101] determined the presence of Osteocalcin using Multiplex assay and concluded that there is significantly higher concentration in symptomatic reversible and irreversible pulpitis *versus* healthy dental pulp tissues. Regarding the levels of TGF-β1, our results are also consistent with Piattelli et al. [165].

The only study that included dental pulp samples collected from TT was that of Suwanchai et al. [7] who determined the presence of MMP-9 in reversibly and irreversibly inflamed pulp tissues (without differentiation between the 2 types of inflammation) compared to healthy teeth, with significantly higher levels in the study group ($p<0.05$).

MMP-7, MMP-9 and Catalase did not show significant differences in acutely and chronically inflamed dental pulp tissues compared to healthy samples. The results of the present study regarding Catalase and MMP-9 concentrations are contradictory with other studies which, regardless of the molecular method of diagnosis (Zymography, RT-PCR, Western-Blot, spectrophotometric method) reported a significantly ($p<0.05$) increased level of these enzymes in the study group represented by inflamed dental pulp samples compared to healthy pulp tissues [7,103,108,112]. Similar to our first study, to the best of our knowledge MMP-7 levels have not been determined in dental pulp inflammation.

Osteocalcin, TGF-β1 and IL-17 showed increased concentrations in the chronically closed pulpitis group compared to acutely inflamed pulp samples ($p=0.001$/$p=0.035$/$p=0.005$ respectively). Increased levels of IL-17 and TGF-β1 have been observed in chronic periapical lesions, TGF-β1 signaling being essential for IL-17 synthesis by Th17 cells [144][145]. Similar mechanisms may explain the increased levels of these molecules in chronic pulpitis. Osteocalcin has been associated with the presence of angiogenesis biomarkers and was detected in areas of tissue fibrosis, these aspects characterizing chronic tissue inflammation [101]. It can be speculated that in chronic pulpitis Osteocalcin has similar mechanisms.

In the case of TT molecular parameters determination and correlation with clinical symptomatology can become useful for the accurate diagnosis of the stage of dental pulp inflammation (reversible/irreversible). Early presentation of the patient with the onset of clinical symptoms is essential to apply less invasive treatments and intercept the inflammation in a reversible stage followed by vital therapies in order to maintain tooth vitality and prevent tooth loss with all the consequences that are followed.

Chapter 8. Biochemical assessment in temporary and permanent teeth with acute pulpitis and possible correlations between biomarkers in healthy and inflamed dental pulp samples

8.1. Introduction

The **aim of the study** was to identify whether there are any differences at molecular level in acute dental pulp inflammation in temporary and permanent teeth. Correlations between biomarkers of inflammation, oxidative stress, ECM degradation and reparation and mineralization of hard tissues in healthy conditions and inflammation, in temporary and permanent dentition, were also assessed.

Specific Objective #1: Determination of TNF-α, IL-2, IL-17, SOD-3, Catalase, MMP-7, MMP-9, Osteocalcin, and TGF-β1 levels in acutely inflamed dental pulp tissues collected from temporary teeth *versus* those obtained from permanent teeth.

Specific objective #2: To establish possible correlations between biomarkers involved in the mechanisms of inflammation (TNF-α, IL-2, IL-17), oxidative stress (SOD-3, Catalase), ECM degradation (MMP-7, MMP-9) and hard tissue reparation and mineralization (Osteocalcin, TGF-β1) in irreversible dental pulp inflammation of temporary and permanent teeth.

Specific Objective #3: Determination of possible correlations between TNF-α, IL-2, IL-17, SOD-3, Catalase, MMP-7, MMP-9, Osteocalcin and TGF-β1 in physiological and pathological (inflamed) conditions of temporary and permanent teeth.

8.2. Material and Method

The study included healthy and irreversibly inflamed dental pulp samples collected from TT and PT of children and adolescents, aged 6-18 years old.

In order to achieve the first objective, 2 study groups with clinical diagnosis of acute total pulpitis were created. Study group 1 included 18 acute inflamed dental pulp tissues obtained from TT while the second study group was represented by acute inflamed dental pulp samples from PT (n=23).

To achieve objective 2, two experimental groups were established and were represented by irreversibly inflamed dental pulp tissues from PT and TT. Study group 1 consisted of acutely and chronically inflamed dental pulp samples (*n*=33) from TT while study group 2 contained 23 acutely inflamed pulp tissues from PT.

To achieve goal 3, we create a total batch of 46 dental pulp samples (*n*=13 healthy, *n*=18 with acute pulpitis, *n*=15 with chronic closed pulpitis) from TT and another one with 42 dental pulp samples from PT (*n*=19 healthy, *n*=23 with acute pulpitis).

Patient's inclusion and exclusion criteria are described in the general methodology chapter.

Teeth exclusion criteria: TT / PT with acute partial pulpitis, TT / PT with necrosis, TT/PT with pathological resorption/perforations/periapical lesions.

Pulp tissue collection, obtaining of pulp lysate and sandwich ELISA analysis are described in the "General methodology" chapter and for each individual dentition in studies 1 and 2 (chapter 6 and 7).

Regarding statistical analysis, Shapiro-Wilk, Mann-Whitney U, Student/Welch tests were used depending on the type and distribution of each variable in order to establish objective 1. Pearson correlation coefficient was used for correlations with independent quantitative variables and parametric distribution, while correlations with independent quantitative variables and non-parametric distribution were established using Spearman's rho correlation coefficient.

8.3. Results

No statistically significant differences were observed between the acutely inflamed dental pulpal tissues collected from PT and TT (Table VIII.14.).

Table VIII.14. Comparison of biomarkers in acutely inflamed dental pulp tissues from PT and TT

Biomarker	Acutely inflamed pulp samples from TT (*n*=18), Mean value ± SD	Acutely inflamed pulp samples from PT (*n*=23), Mean value ± SD	Statistical significance (*p*)
IL-2 (pg/mg prot.)	108 ± 32.77	113.21 ± 29.14	0.973**
IL-17 (pg/mg prot.)	130.71 ± 12.38	138.04 ± 16.54	0.167**
TNF-α (pg/mg prot.)	57.71 ± 10.78	54.435 ± 11.195	0.125***
MMP-7 (ng/mg prot.)	2.25 ± 0.69	2.3 ± 0.85	0.867***
MMP-9 (ng/mg prot.)	1.44 ± 0.53	1.496 ± 0.5	0.763**
SOD-3 (ng/mg prot.)	27.45 ± 8.31	26.32 ± 7.71	0.750**
Catalase (U/mg prot.)	2.59 ± 0.75	2.67 ± 0.74	0.466**
Osteocalcin (ng/mg prot.)	5.87 ± 3	7.97 ± 4	0.305**
TGF-β1 (pg/mg prot.)	18.07 ± 4.5	23.16 ± 11.79	0.185***

(prot.=protein, * Welch T-Test, ** Student T-Test, *** Mann-Whitney U Test)

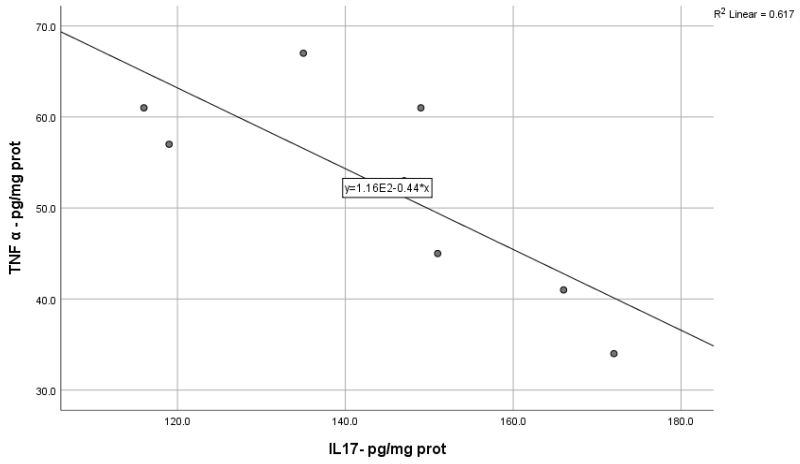


Figure 8.10. Correlation between TNF-α and IL-17 in acutely and chronically inflamed pulp samples from TT

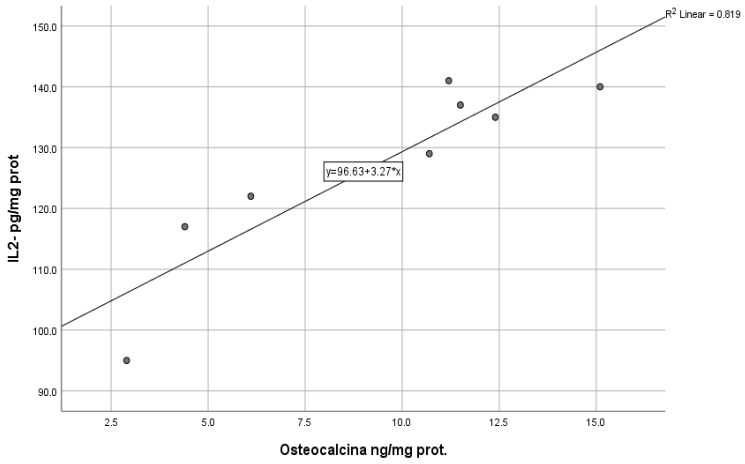


Figure 8.11. Correlation between IL-2 and Osteocalcin in acutely and chronically inflamed pulp samples from TT

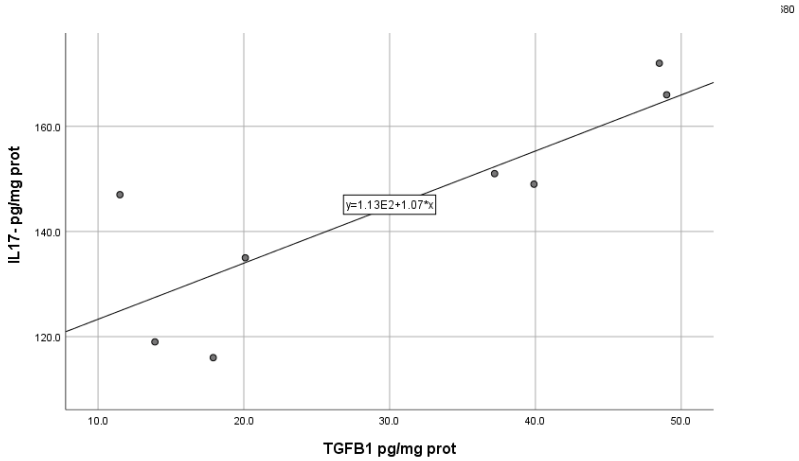


Figure 8.12. Correlation between IL-17 and TGF-β1 in acutely and chronically inflamed pulp samples from TT

Figures 8.10.-8.12. present the correlations between biomarkers in dental pulp samples with acute and chronic irreversible inflammation from TT. The distribution of variables is normal according to Shapiro-Wilk test (p>0.05). Most of the analyzed correlations are not statistically significant with some exceptions:

- The correlation between TNF-α and IL-17 is significant and strongly negative (p=0.021, R=-0.786), showing that higher TNF-α levels are more frequently associated with lower IL-17 levels and vice-versa.
- The correlation between IL-2 and Osteocalcin is significant and positive (p=0.002, R=0.905) showing that higher IL-2 levels are more frequently associated with higher Osteocalcin levels and vice-versa.

- The correlation between IL-17 and TGF-β1 is significant and strongly positive (p=0.012, R=0.825) showing that higher IL-17 values are more frequently associated with higher TGF-β1 values and vice-versa.

Regarding the analyzed correlations between biomarkers in the total group of pulp samples from TT, the distribution of variables is non-parametric according to Shapiro-Wilk test (p<0.05). Significant correlations were observed in the following situations:

- The correlations between TNF-α and IL-2 (p<0.001, R=0.811), TNF-α and IL-17 (p=0.020, R=0.544), TNF-α and SOD-3 (p<0.001, R=0.819), TNF-α and Osteocalcin (p=0.012, R=0.579) and between TNF-α and TGF-β1 (p=0.008, R=0.602) are significant and positive of high or very high degree, showing that elevated TNF-α levels are more frequently associated with higher IL-2, IL-17, SOD-3, Osteocalcin and TGF-β1 levels.
- The correlations between IL-2 and IL-17 (p=0.001, R=0.707), IL-2 and SOD-3 (p<0.001, R=0.806), IL-2 and Osteocalcin (p=0.001, R=0.721) and between IL-2 and TGF-β1 (p=0.016, R=0.558) are significant and positive of high or very high degree, showing that higher IL-2 values are more frequently associated with higher levels of IL-17, SOD-3, Osteocalcin and TGF-β1.
- The correlations between IL-17 and SOD-3 (p=0.004, R=0.639), IL-17 and Osteocalcin (p=0.006, R=0.624) and between IL-17 and TGF-β1 (p=0.037, R=0.494) are significant and positive of moderate or high degree, showing that higher IL-17 values are more frequently associated with higher values of SOD-3, Osteocalcin and TGF-β1.
- The correlations between SOD-3 and Osteocalcin (p=0.015, R=0.562) and between SOD-3 and TGF-β1 (p=0.025, R=0.526) are significant and high-grade positive, showing that higher values of SOD-3 are more frequently associated with higher levels of Osteocalcin and TGF-β1.
- The correlation between Catalase and Osteocalcin (p=0.014, R=0.567) is significant and high-grade positive, showing that higher Catalase values are more frequently associated with higher Osteocalcin values.
- The correlation between Osteocalcin and TGF-β1 (p=0.005, R=0.636) is significant and highly positive, showing that higher Osteocalcin values are more frequently associated with higher TGF-β1 values.

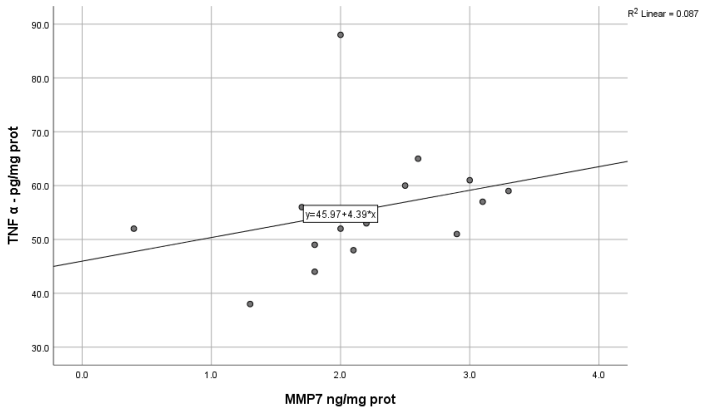


Figure 8.29. Correlation between TNF-α and MMP-7 in acutely inflamed pulp samples from PT

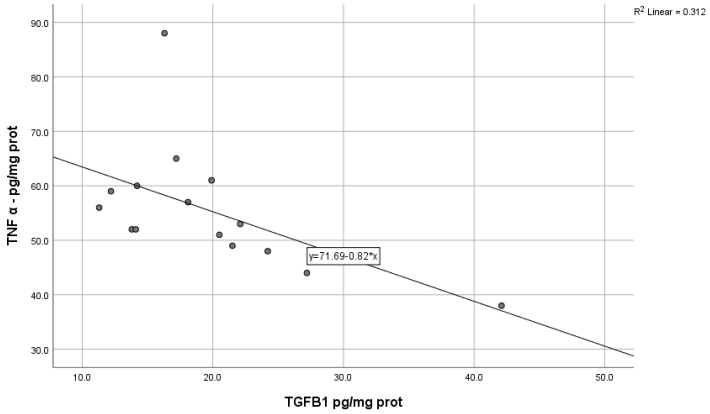


Figure 8.30. Correlation between TNF-α and TGF-β1 in acutely inflamed pulp samples from PT

Figures 8.29. - 8.30. represent the correlations between biomarkers in acutely inflamed pulp samples from PT. The distribution of the variables is normal according to Shapiro-Wilk test (p>0.05), except TNF-α and TGF-β1 (p<0.05). Most of the analyzed correlations are not statistically significant with some exceptions:

- The correlation between TNF-α and MMP-7 is significant and moderately positive (p=0.045, R=0.524) showing that higher TNF-α values are more frequently associated with higher MMP-7 values and vice-versa.
- The correlation between TNF-α and TGF-β1 is significant and moderately negative (p=0.019, R=-0.595) showing that higher TNF-α values are more frequently associated with lower TGF-β1 values and vice-versa.

The analyzed correlations between biomarkers in the total group of pulp samples from PT show that the distribution of variables is non-parametric according to Shapiro-

Wilk test (p<0.05), except for MMP-9, MMP-7 and Catalase (p>0.05). Significant correlations were observed between:

- TNF-α and IL-2 (p<0.001, R=0.715), TNF-α and IL-17 (p=0.001, R=0.651), TNF-α and MMP-7 (p<0.001, R=0.670), TNF-α and SOD-3 (p=0.001, R=0.624), TNF-α and Osteocalcin (p=0.006, R=0.543) and between TNF-α and TGF-β1 (p=0.038, R=0.426) are significant and positive of moderate or high degree, showing that higher TNF-α values are more frequently associated with higher values of IL-2, IL-17, MMP-7, SOD-3, Osteocalcin and TGF-β1.
- IL-2 and IL-17 (p<0.001, R=0.704), IL-2 and SOD-3 (p=0.001, R=0.638), IL-2 and Osteocalcin (p<0.001, R=0.664) and between IL-2 and TGF-β1 (p=0.002, R=0.590) are significant and high-grade positive, showing that elevated IL-2 values are more frequently associated with higher levels of IL-17, SOD-3, Osteocalcin and TGF-β1.
- IL-17 and MMP-7 (p=0.010, R=0.513), IL-17 and SOD-3 (p<0.001, R=0.823), IL-17 and Catalase (p=0.028, R=0.448), IL-17 and Osteocalcin (p=0.001, R=0.620) and between IL-17 and TGF-β1 (p=0.002, R=0.604) are significant and positive of moderate or high degree, showing that elevated levels of IL-17 are more frequently associated with higher levels of MMP-7, SOD-3, Catalase, Osteocalcin, and TGF-β1.
- MMP-9 and Catalase (p=0.034, R=0.435) is significant and positive of moderate degree, showing that higher levels of MMP-9 are more frequently associated with higher Catalase values.
- MMP-7 and SOD-3 (p=0.008, R=0.531), MMP-7 and Catalase (p=0.044, R=0.415) are significant and positive of moderate degree, showing that higher values of MMP-7 are more frequently associated with higher levels of SOD-3 and Catalase.
- SOD-3 and Osteocalcin (p=0.001, R=0.628) and between SOD-3 and TGF-β1 (p=0.001, R=0.647) are significant and high-grade positive, showing that higher values of SOD-3 are more frequently associated with higher levels of Osteocalcin and TGF-β1.
- Osteocalcin and TGF-β1 (p=0.015, R=0.492) is significant and moderately positive, showing that higher Osteocalcin values are more frequently associated with higher TGF-β1 values.

8.4. Discussions

Although there are notable differences between TT and PT that are followed by various clinical and therapeutical implications [177], based on the results of this study we can speculate that at molecular level the symptomatic irreversible inflammatory process of the dental pulp does not differ between the 2 dentitions.

An inversely proportional relationship between TNF-α and IL-17 has been observed in the irreversible dental pulp inflammation of TT. Both TNF-α and IL-17 are proinflammatory cytokines and the results may suggest an antagonistic role in irreversible pulpitis of TT, in contrast to other studies that support the existence of a synergistic role between these 2 [146]. The highly positive correlation of IL-2 with Osteocalcin and IL-17 with TGF-β1 may suggest the tendency of pulp tissue to limit inflammation, which is exacerbated by elevated interleukin concentrations, with the concomitant release of Osteocalcin and TGF-β1 that may contribute to the healing through tertiary dentine formation.

In acutely inflamed pulp samples from PT, TNF-α has been positively correlated with MMP-7 which confirms the important role of cytokines in regulating MMP expression. TNF-α high concentrations can influence the increased activity of MMP-7 in pulpitis. MMP-7 in turn can have an impact on the inflammatory process by releasing increased amounts of TNF-α. An inversely proportional relationship was noted between TNF-α and TGF-β1 in the acute pulpitis group of PT, TNF-α being a proinflammatory cytokine that mediates dental pulp inflammation, whereas TGF-β1 is a growth factor released from dentine with role in ECM protein synthesis and dentinogenesis. Thus, low TNF-α concentration and increased TGF-β1 synthesis suggest a tendency to reduce dental pulp inflammation and the possibility of pulpal healing and repair (and vice-versa).

Regarding the correlations of biomarkers in normal and irreversibly inflamed temporary and permanent teeth, most of them were positive suggesting an interconnection between mechanisms of inflammation, oxidative stress, ECM degradation and tissue reparation and mineralization, both in healthy and pathological conditions.

In the total group of PT were also reported positive correlations between enzymes and other molecules. A more intense enzymatic activity can be observed in the acute irreversible pulpitis of PT which could be explained by the establishment of a more accurate clinical diagnosis (older age of included patients, more concrete and correct information related to symptoms, more precise vitality tests and tooth percussion, CNS maturity) compared to TT

(younger age, underdeveloped language and CNS, higher degree of fear). Dental pulp inflammation at molecular level in TT may already be at a very advanced stage with progression to necrosis, influenced also by regressive changes that occur physiologically and may be associated with the destruction or depletion of certain enzymes.

9. Conclusions and personal contributions

9.1. Conclusions

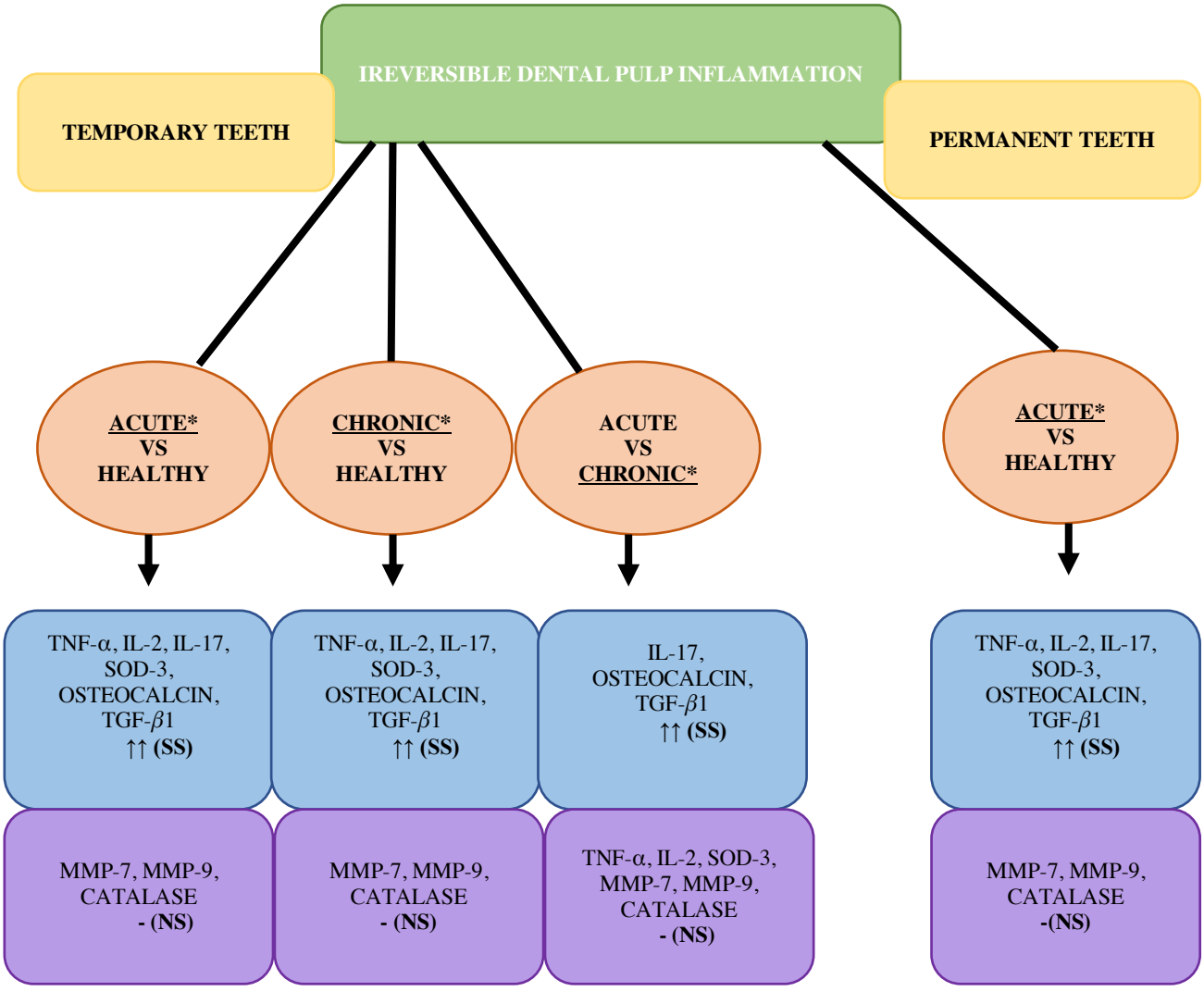
- An up-regulation of TNF-α, IL-2, IL-17, SOD-3, Osteocalcin and TGF-β1 can be observed in irreversibly inflamed dental pulp tissues from PT and TT (Figure 9.1.1.).
- Elevated levels of certain biomarkers may play an important role in the pulpal inflammatory processes of temporary and permanent teeth of children and adolescents.
- IL-17, Osteocalcin and TGF-β1 showed increased concentration in the chronic pulpitis group compared to the acute inflamed pulp samples of TT and can be considered potential biomolecules in differentiating the two types of inflammation (Figure 9.1.1.).
- Lack of significant differences in MMP-7, MMP-9 and Catalase levels between control and study groups in both TT and PT may suggest an advanced phase of irreversible dental pulp inflammation to the stage of necrosis associated with decreased production/destruction of these enzymes.
- Less correlations between biomarkers in the study groups of irreversible dental pulp inflammation in both dentitions indicate the need for additional research in the field to accurately establish the role and mechanisms of these molecules in dental pulp inflammation.
- Presence of all biomarkers in every single pulp tissue sample and significant correlations between most of them in healthy and inflammatory conditions, suggest their major role and involvement in pulp tissue homeostasis mechanisms and bacterial inflammation.
- The absence of significant differences in acute pulpitis of TT compared to acute dental pulp inflammation of PT indicates the presence of similar molecular

mechanisms in the 2 dentitions although between TT and PT exist morpho-structural differences.

- The degree of cooperation of pediatric patients, the more difficult communication and expression of their feelings, and also the uncertain results of clinical and paraclinical tests are key factors in creating the conditions for pulp tissue collection and possible causes of lack of studies based on biomarkers determination in dental pulp samples from children and adolescents.
- Traditional diagnostic clinical tests used to determine the presence of dental pulp inflammation are strictly subjective and may have variable interpretations, while the level of professional expertise varies and discrepancies may occur in their interpretation, especially in children and adolescents.
- The findings of the present studies suggest the need for molecular diagnostic methods that could be associated with clinical data to establish a precise diagnosis, ideally in the early stages of inflammation.
- Establishing protocols for accurate diagnosis by combining clinical data with molecular methods can help choosing the right dental therapy avoiding over-/under-treatment.
- To put in practice molecular diagnostic methods, it is important to establish threshold values of biomarkers depending on the type of inflammation (reversible / irreversible) as well as finding feasible tools to use in daily practice.
- The small number of pulp tissue samples used, and the dynamics of the pulp inflammatory process may be causal factors in the diversity of results compared to other studies.
- Identification of new molecules for more accurate diagnosis assessment and implementation of less invasive treatments should be taken into consideration especially in the case of dental pulp inflammatory processes in children and adolescents.
- Need of new methods to assess dental pulp inflammation at the molecular level through less invasive methods, such as saliva collection from patients with pulpitis and association with clinical data.
- Postponement of dental treatments, check-ups and late presentation for treatment in advanced stages of inflammation lead to the need for radical therapies such as

complete removal of pulp tissue with multiple consequences in both teeth (more fragile teeth with higher risk of fracture, early loss, misalignment, malocclusions).

- Early diagnosis followed by less invasive vital pulp therapies are the gold standard, especially for young permanent teeth in order to continue apexogenesis.
- More research in the field is needed, particularly in case of pulpitis in children and adolescents where lack of studies has been observed.



(*indicates statistically significant (SS) differences, NS=not statistically significant differences)

Figure 9.1.1. General conclusions

9.2. Personal contributions

The literature is characterized by lack of studies regarding the molecular diagnosis of dental pulp inflammation in children and adolescents, so the determinations realized in this research, as well as the patients from whom pulp tissue samples were collected, give this work an innovative and original character.

The personal contributions of this research are:

The results of **first study described in chapter 6** showed elevated levels of TNF-α, IL-2, IL-17, SOD-3, Osteocalcin and TGF-β1 in the acute irreversible pulpitis of PT obtained from children and adolescents which has not been studied so far (except SOD, but with uncertain results). Also, in this study was studied for the first time the presence and concentration of MMP-7 in irreversible dental pulp inflammation in children and adolescents. In addition, biomarkers such as Osteocalcin and TGF-β1 which have been evaluated in a very limited number of studies in the past, were determined in acutely inflamed and healthy pulp samples from PT. Molecular diagnosis using these biomarkers may be useful in association with patient's symptoms and clinical signs and can be used in early diagnosis of dental pulp pathology (reversible stage) followed by less invasive treatments with partially removal of pulp tissue in order to maintain tooth vitality followed by apexogenesis in young PT.

The study in **Chapter 7** was based on the assessment of various biomarkers in acute pulpitis, chronic pulpitis and healthy dental pulp tissue collected from children and adolescents' TT, a topic which was not studied in the literature to the best of our knowledge. The present study aimed to show the importance of applying molecular methods in the case of TT dental pulp inflammation and the results show that TNF-α, IL-2, IL-17, SOD-3, Osteocalcin and TGF-β1 can be used as potential biomarkers for the diagnosis of acute and chronic pulpitis of TT. Also, for the first time several biomarkers were studied comparatively in acute and chronic pulpitis of temporary dentition, and the findings of our study suggest a major role of IL-17, Osteocalcin and TGF-β1 in chronic pulpitis. This study also highlights the importance of establishing molecular tools for early diagnosis in an effort to avoid radical treatments followed by potential tooth loss.

Chapter 8 aimed to determine the differences in TNF-α, IL-2, IL-17, SOD-3, Catalase, MMP-7, MMP-9, Osteocalcin and TGF-β1 levels in the acute pulpitis samples from TT and PT. Also, this aspect has not been studied so far to the best of our knowledge

and the results of the study suggest similar mechanisms of inflammation, oxidative stress, ECM degradation and mineralization and reparation of tissues in both dentitions. We did not find any studies evaluating possible correlations between biomarkers in inflamed and healthy dental pulp tissues from children and adolescents. The positive correlations between various biomarkers both in healthy and inflamed dental pulp samples show the existence of interconnections between the studied biomarkers in both dentitions. The correlations observed in the irreversible dental pulp inflammation in TT and PT may suggest the existence of antagonistic roles between certain biomarkers and sometimes synergistic roles with reduction of inflammation mechanisms and stimulation of the reparative processes.

More research is needed to elucidate the precise role of the studied biomarkers and other potential biomolecules in inflammatory dental pulp conditions in PT and TT from children and adolescents.

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Participation in scientific events

- **Kritikou, K.;** Ripzky Totan, A.; Imre, M.; Spinu, T.-C.; Tanase, M.; Greabu, M. A correlation study applied to biochemical biomarkers of inflammation, oxidative stress and extracellular matrix in human dental pulp. "Carol Davila" University of Medicine and Pharmacy Congress, Bucharest, 25-27th November 2021- Diploma of Excellence in Young Researcher Session-Dental Medicine field
- **Kritikou, K.;** Greabu, M.; Ripzky Totan, A.; Miricescu, D.; Tanase, M. Assessment of biochemical markers in young human dental pulp. 16th EAPD Congress. Lisbon, 15-18th June 2022.

- Enasescu, D.; Ripzky Totan, A.; **Kritikou, K.;** Miricescu, D.; Stanescu, I.; Greabu, M. Assessment of mineralization, oxidative stress, and inflammation mechanisms in primary teeth's pulp. IADR Congress, 100th General Session & Exhibition. 20-25th June 2022, virtual session.

Other awards

- PRECISI2021 Competition: Award for the article „ILs and MMPs levels in inflamed human dental pulp: A systematic review", published in Molecules Journal (eISSN: 1420-3049), I.F. : 4.411, Prize: 2000 RON.