The influence of the tooth preparation finish line position on the expression of matrix metalloproteinase-9 and the presence of periodontopathogens in the gingival crevicular fluid

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Abstract. – **OBJECTIVE:** The objective of the study was to determine the concentration of matrix metalloproteinase 9 (MMP-9) and changes in the presence of periodontopathogens in the gingival crevicular fluid before and after tooth preparation with the subgingival and equigingival finish line position.

PATIENTS AND METHODS: The clinical prospective study included 20 subjects with an indication for upper canine preparation, with the subgingival (group 1) and equigingival finish line (group 2). Samples were taken in four observation intervals: 5 minutes before (control samples), as well as 15 minutes, 24 and 72 hours after tooth preparation (experimental samples). Measurement of MMP-9 was done using Enzyme-linked Immunosorbent Assay (ELISA). The presence of bacteria in the gingival fluid was proven by the Polymerase chain reaction (PCR) analysis.

RESULTS: The MMP-9 values did not differ statistically significantly between the groups (p=0.524). The MMP-9 values showed a statistically significant difference in the given observation period (p<0.001) with a significant linear increase in values (p<0.001). A significant quadratic trend recorded a decrease in the MMP-9 values 15 minutes after preparation, and an increase 24 hours after preparation, without a significant difference in the interaction between groups (p=0.392). After preparation, a significant difference in the presence of periodontopathogens was confirmed, i.e., a decrease in the presence of *Prevotella intermedia* (p=0.025) and *Tannerella forsythia* (p=0.016) in group 1, and an increase in the presence of *Aggregatibacter actinomycetemcomitans* in both groups (p=0.029, p=0.026).

CONCLUSIONS: The study is a good basis for determining the influence of tooth preparation on gingival inflammation, with therapeutic (choice of preparation technique) and preventive significance regarding the protection of the periodontal tissue from possible iatrogenic damage.

Key Words:
Tooth preparation, Finish line position, Matrix metalloproteinase-9, Periodontopathogens.

Introduction

Adherence to the rules of tooth preparation is vital for the durability and stability of fixed prosthetic restorations[1]. The success of therapy with fixed restorations is conditioned by adequate preparation between the prepared and the unprepared tooth structure (finish line) to ensure an optimal fit of the artificial crown on the prepared tooth, without a marginal gap and consecutive caries or periodontitis[2]. The shape and the position of the finish line are a condition for preserving periodontal health[3,4]. The position of the finish line can be below (subgingival), at the level (equigingival), and above the level of the gingiva (supragingival). Ceramic and Porcelain-Fused-to-Metal (PFM) crowns with chamfers exhibited significantly better internal adaptation than those with rounded shoulders[5].

The importance of preserving the gingival tissue during tooth preparation and possible inflammation of the gingiva after this daily dental procedure was described by Newcomb[6] back in 1974, who pointed out the disadvantages of the subgingival finish line position. Regardless of the proven better biological characteristics of the equigingival location, the subgingival finish line is indicated in about 50% of clinical cases (caries

and hypersensitivity in the tooth neck area, earlier subgingival preparation, improved retention, crown resistance, and better esthetics)[7]. The area determined by the gingival margin of the artificial crown and the edge of the alveolar bone, which does not cause bone resorption, is called the biological zone. If the gingival margin of the prosthetic replacement is placed no more than 0.5 mm below the gingival margin, it will cause no alveolar bone resorption[8].

The most common iatrogenic effects of tooth preparation are mechanical damage and recession of the marginal gingiva, as well as acute and chronic inflammation of the gingival and periodontal tissue[9]. Mechanical tissue trauma is characterized by the detachment of the gingival epithelium and reattachment during tissue healing. However, it can lead to histological changes in the connective tissue that cause apical displacement of the gingiva[10].

The durability of fixed restorations depends on the state of the periodontium. Therefore, iatrogenic inflammation of the gingiva caused by tooth preparation should be repaired in time[11,12]. On the other hand, research on this topic is scarce in the literature, and the comparison of individual parameters that accompany gingival damage during tooth preparation for the acceptance of fixed restorations is almost non-existent. Moreover, neither the change in the oral flora after this common prosthetic procedure nor the change in the expression of inflammation markers that accompanies it was investigated. One of the inflammation markers on the periodontal tissue is the group of matrix metalloproteinases (MMPs).

MMPs represent a structurally related, but genetically different, superfamily of proteases. They actively participate in the physiological development and remodeling, but also in pathological inflammation and malignant tissue destruction[13]. The group of MMPs can degrade almost all components of the extracellular matrix and cell membranes, so their excessive activity destroys the periodontal tissue[14,15]. The effect of MMPs is achieved by regulating the effects of various bioactive non-matrix substrates, such as growth factors, immune mediators, cytokines, chemokines, etc., thereby indirectly controlling pro- and anti-inflammatory processes[16,17].

Research[13,18,19] by numerous authors shows that polymorphonuclear leukocytes and monocytes, i.e., macrophages, migrate to sites of inflammation in response to the effect of present periodontopathogens. At these sites, they release matrix metalloproteinase 9 (MMP-9) through degranulation, which is regulated by the expression of the MMP-9 gene, located on chromosome 20q11.2-13.1. MMP-9 has been found to regulate the release of certain mediators during the early stages of inflammation, including interleukins (IL-1, -6 and -8), and prostaglandins[20,21].

MMP-9 is usually secreted in combination with a specific inhibitor, tissue inhibitor of matrix metalloproteinase (TIMP-1). An imbalance between MMP-9 and TIMP-1 leads to excessive tissue degradation, common in chronic inflammatory diseases including chronic periodontitis[22,23]. Ingman et al[24] demonstrated elevated levels of TIMP-1 in patients with periodontitis compared to the control group.

Microorganisms of the subgingival plaque are the cause of destructive changes in the periodontal tissue, i.e., they cause initial gingivitis to develop into more severe cases of periodontitis[25]. Long-term and effective control of the subgingival plaque is crucial for stopping progressive processes on the supporting tooth apparatus. The presence of periodontopathogens in shallow pockets is a sign of infection and their persistence in deep periodontal pockets is a sign of active or exacerbated chronic periodontosis[26].

Bacteria responsible for periodontal diseases are shown in Table I[27].

Periodontopathogens from the Aa complex, as well as the red and orange complexes, are responsible for the occurrence of periodontal diseases. Therefore, reducing their number (during treatment) can be an effective parameter in controlling therapy progress and long-term stabilization of

Table I. Association of periodontal pathogens with bacterial complexes.

Complexes	Pathogen Strain
Aa-complex	Aggregatibacter actinomycetemcomitans
Red complex	Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia
Orange complex	Prevotella intermedia, Peptostreptococcus (Micromonas) micros, Fusobacterium nucleatum
Orange-associated complex	Eubacterium nodatum
Green complex	Capnocytophaga gingivalis

• Very highly pathogenic - Aa-complex. • Highly pathogenic - red complex. • Highly to moderately pathogenic - orange and orange-associated complexes. • Moderately pathogenic - green complex.

periodontal diseases[28-30]. The subgingival biofilm, in which *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* are present, either individually or in combination, is associated with a high risk for periodontal disease progression[31-33]. The periodontopathogens *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia*are are more often detected in deep (>5 mm) than in shallow (<4 mm) periodontal pockets[31]. Socranski et al[27] named this group of bacteria "the red complex".

The study assumed that tooth preparation mechanically damages the gingival tissue. This applies especially in the case when the finish line is positioned in the area of the gingival sulcus and thus causes a change in the oral flora in the gingival sulcus and the inflammation of the gingiva.

The aim of the study was to determine the concentration of matrix metalloproteinase 9 and monitor changes in the presence of reference microorganisms (*Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Tannerella forsythia, Porphyromonas gingivalis*) in the gingival crevicular fluid before and after tooth preparation with subgingival and equigingival finish line positions.

Patients and Methods

The study protocol was approved by the Ethics Committee of the Faculty of Medicine (Decision No.: 68/5-2019-3EO). All subjects were informed of the purpose of the study and signed the Informed Consent statement.

Subjects and Clinical Procedure

The clinical prospective study included 20 subjects with an indication for upper canine preparation in order to make a PFM. Subjects of both sexes, without systemic and infectious diseases, non-smokers, aged 30-40, without pathological changes in the soft tissues of the oral cavity, with healthy periodontium, without carious lesions and prosthetic restorations were included in the study. The subjects did not take antibiotics for two months before the study. Immediately before the intervention, the gingival index and the gingival bleeding index on the tooth indicated for preparation amounted to 0, suggesting the absence of inflammation[34].

Regarding the position of the finish line, the subjects were divided into two groups: in group 1, the preparation was performed with the finish line positioned 0.3 to 0.5 mm below the level of the gingival margin (subgingival), whereas in group 2 the finish line was localized at the level of the gingival margin (equigingival). The position of the finish line was determined according to clinical criteria.

Following all standards, tooth preparation was performed by a single therapist, with maximum preservation of the margin of the gingiva, and lasted for 15 minutes. A chamfer finish line was formed because this type of finish line for a PFM restoration enables less removal of the dental tissue, marginal integrity, protection of periodontal tissues, great longevity of prosthetic restorations and esthetics[35].

Gingival fluid content samples for biochemical analysis and molecular genetic analysis were collected using six sterile paper points (#30, Spident CO., Gojan-Dong, Korea). Paper points were applied to the gingival sulcus of the treated tooth, three each from the labial and palatal side, for 20 seconds. Sampling for the Polymerase chain reaction (PCR) analysis involved taking 30 samples in both groups of subjects, for all four observation intervals. The points were placed in sterile plastic test tubes (Eppendorf) with a volume of 1.5 ml³ and stored at a temperature of -70°C until analysis.

Control samples were taken 5 minutes before tooth preparation, whereas experimental samples were taken 15 minutes, 24 and 72 hours after tooth preparation (four observation intervals).

The clinical procedure of making temporary crowns, taking an impression, and making a PFM restoration continued after taking the third observation interval (72 h), considering that each of the mentioned procedures can influence the observed inflammation[36-38].

Enzyme-Linked Immunosorbent Assay (ELISA)

All samples were defrosted to a temperature of 4°C and then centrifuged at 1,000 rpm for 20 minutes (Eppendorf MiniSpin®; Hamburg, Germany).

The concentration of MMP-9 was determined using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) with double antibodies. The MMP-9 Human ELISA Kit (Invitrogen - Thermo Fisher Scientific, Waltham, MA, USA) detection range between 0 pg/mL - 1,500 pg/mL Hu MMP-9, sensitivity <10 pg/mL, was used.

The tests were performed following the manufacturer's instructions.

The values were measured spectrophotometrically on the ELISA reader Rayto RT 2100C Microplate Reader (China), at a wavelength of 450 nm.

A standard curve was constructed using standards from the ELISA kit. The concentration of MMP-9 in the samples was determined by comparing the Optical Density (O.D.) of the samples on the standard curve.

Polymerase Chain Reaction (PCR)

The presence of the genome of periodontopathogenic microorganisms in the gingival fluid samples was proven by the polymerase chain reaction (PCR).

Prior to DNA isolation, the samples were prepared by adding 300 µl of 50 mmol NaOH and vortexed for 10 seconds. The samples were then heated in a thermomixer for 5 minutes at 950°C, 30 µl of 1 M trisHCL (pH=8) was added, and after that, they were centrifuged at 13,000 revolutions for 2 minutes (Eppendorf MiniSpin®; Hamburg, Germany).

The isolation of possibly present bacterial DNA was performed by treating the samples with proteinase K (MBI Fermentas, Vilnius, Lithuania) at a temperature of 56°C for 30 minutes. The samples were then heated at a temperature of 95°C for 15 minutes to inactivate the enzyme. The treated material was stored at -20°C until the PCR procedure.

The gingival fluid samples were examined for the presence of four bacteria: *Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Tannerella forsythia,* and *Porfiromonas gingivalis.*

Positive controls included the DNA obtained from reference strains of microorganisms. Distilled water was used for negative controls, the DNA sample was omitted.

The PCR analysis was performed in a 25 µl mixture containing: 1X PCR buffer (MBI Fermentas, Vilnius, Lithuania), 1.5 mM MgCl2, 0.2 mM deoxyribonucleoside-triphosphates (dNTPs), 0.375 µM of each species-specific primer, 1 unit of Taq DNA polymerase (MBI Fermentas), and 5 µl of bacterial DNA isolate[37].

Known primer sequences were used for the application of the PCR technique. The sequences of all primers and the expected amplicon lengths are shown in Table II.

The PCR analysis was performed in a thermal cycle (Peqlab, PeqSTAR 2X; Erlangen, Germany) starting with initial denaturation (95°C for 3 minutes), followed by a cycle of 35 repetitions: denaturation (94°C for 45 seconds), hybridization (55°C for 60 seconds), elongation (72°C for 60 seconds), and final elongation (72°C for 5 minutes).

The PCR products were analyzed by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide, and finally visualized and photographed after exposure to ultraviolet (UV) light.

Statistical Analysis

Using descriptive statistical methods, continuous variables are presented as arithmetic mean ± standard deviation, whereas categorical variables are presented as a number (percentage). For continuous variables, the normality of distribution was assessed using the Shapiro-Wilk test. The difference in MMP values between the groups and in the observed time was evaluated by employing the analysis of variance for repeated measurements. The Fisher's exact probability test was used to analyze the difference in the frequency of causes between the groups, whereas the Cohran's and McNemar's tests were used for the difference in the frequency of causes in the observed period. Statistical hypotheses were tested at the level of statistical significance (alpha level) of 0.05. The software program SPSS Statistics 22 (IBM Corp., Armonk, NY, USA) was used for the statistical processing of the results.

Results

For MMP-9 values obtained from 20 subjects, the difference between the two groups was observed, as well as the difference in values during the observed intervals, following a linear and quadratic trend in the given observation period (Figure 1).

The average values of MMP-9 in the subjects in group 1 were the following: 20.9±1.0 pg/mL

Table II. Primers used for the PCR analysis, hybridization temperatures, and expected amplicon lengths.

Bacterium	Primer sequences (5′-3′)	Hybridization temperature	Amplicon length
Aggregatibacter actinomycetemcomitans	CAC TTA AAG GTC CGC CTA CGT GC	55°C	500 bp
Prevotella intermedia	GTT GCG TGC ACT CAA GTC CGC C	55°C	259 bp
Tannerella forsythia	GTA GAG CTT ACA CTA TAT CGC AAA CTC CTA	55°C	600 bp
Porphyromonas gingivalis	CAA TAC TCG TAT CGC CCG TTA TTC	55°C	400 bp
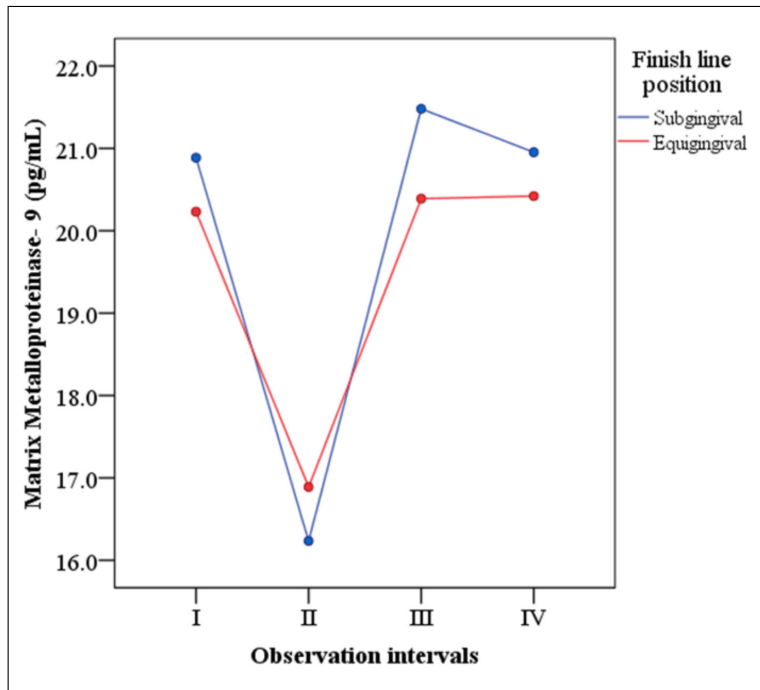
Figure 1. Values of MMP inflammatory markers between groups and the observation intervals (pg/mL). I – before preparation. II – 15 minutes after preparation. III – 24 h after preparation. IV – 72 h after preparation.

before preparation, 16.2±3.5 pg/mL 15 minutes after preparation, 21.5±0.6 pg/mL 24 h after preparation, and 21.0±1.2 pg/mL 72 h after preparation. In group 2, the average MMP-9 values were the following: 20.2±2.1 pg/mL before preparation, 16.9±2.0 pg/mL 15 minutes after preparation, 20.4±2.2 pg/mL 24 h after preparation, and 20.4±2.1 pg/mL 72 h after preparation. The values of MMP-9 did not differ statistically significantly regarding the groups of subjects, i.e., the position of the finish line, subgingival vs. equigingival (F=3,982.8, *p*=0.524).

The MMP-9 values, determined in four measured intervals, show a statistically significant difference in the given observation period (F=30.561, *p*<0.001). In the examined interval, there was a statistically significant linear trend of an increase in the MMP-9 values (F=19.010, *p*<0.001). Furthermore, there was a significant quadratic trend with a decrease in the MMP-9 values 15 minutes after preparation, which in the further course of the examined interval exhibited a significant increase. In other words, the values 72 hours after preparation were slightly higher than the values before preparation.

The values of MMP-9 differed in the observed period, but the interaction of these changes did not differ significantly between the groups (F=0.977, *p*=0.392). Changes in MMP-9 values between measurements during the observed period were identical for both groups of subjects.

Table III shows the difference in the frequency of certain periodontopathogens observed between the groups (subgingival to equigingival finish line) for each observation interval and whether the change in the presence of bacteria was significant during the observation period. The Fisher's test was used to analyze the difference in the frequency of causative agents between the groups in the observed intervals. For each group and bacterium, Cochran's test was performed to test the difference in the presence of periodontopathogens during the observation period and determine whether there was an increase or decrease in causative agents in any of the intervals.

In the subjects in group 2 (with the equigingival finish line position), statistically significantly more frequent pathogens in the gingival fluid were *Aggregatibacter actinomycetemcomitans* confirmed 72 hours after preparation (IV measurement) (*p*=0.023) and *Prevotella intermedia* in the sample obtained 15 minutes after preparation (II measurement) (*p*=0.033). Differences in frequency in other observation intervals, including other causative agents (*Tannerella forsythia, Porphyromonas gingivalis*), concerning the position of the finish line during the preparation, were not significant for any of the observed intervals.

The presence of periodontopathogens changed during the observed period in both groups of subjects, but only for certain causative agents.

Table III. The presence of bacteria in the gingival crevicular fluid in the examined groups and different observation intervals.

Bacteria (Observation period)	Finish line position		Fisher's test (*p*-value)
	Subgingival 10 (50.0%)	Equigingival 10 (50.0%)	
Aggregatibacter actinomycetemcomitans			
Before preparation	2 (20.0)	6 (60.0)	0.170
15 min after preparation	1 (10.0)	2 (20.0)	1.00
24 h after preparation	5 (50.0)	7 (70.0)	0.650
72 h after preparation	2 (20.0)	8 (80.0)	0.023*
Cochran's test (p-value)	0.029*	0.026*	
Prevotella intermedia			
Before preparation	5 (50.0)	6 (60.0)	1.00
15 min after preparation	0 (0.0)	5 (50.0)	0.033*
24 h after preparation	2 (20.0)	6 (60.0)	0.170
72 h after preparation	1 (20.0)	4 (40.0)	0.121
Cochran's test (p-value)	0.025*	0.697	
Tannerella forsythia			
Before preparation	9 (90.0)	10 (100.0)	1.00
15 min after preparation	3 (30.0)	5 (50.0)	0.650
24 h after preparation	7 (70.0)	7 (70.0)	1.00
72 h after preparation	7 (70.0)	7 (70.0)	1.00
Cochran's test (p-value)	0.016*	0.063	
Porphyromonas gingivalis			
Before preparation	4 (40.0)	2 (20.0)	0.628
15 min after preparation	1 (10.0)	1 (10.0)	1.00
24 h after preparation	3 (30.0)	3 (30.0)	1.00
72 h after preparation	1 (10.0)	2 (20.0)	1.00
Cochran's test (p-value)	0.234	0.721	

Data are presented as number (percentage),*- statistically significant.

The difference in the frequency of bacteria in the observed period was significant for subjects in group 1 with the subgingival finish line position, namely for *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, and *Tannerella forsythia*. In the case of *Aggregatibacter actinomycetemcomitans* (p=0.029), there was an increase in the presence of the causative agent in the 24-hour compared to the 15-minute interval after preparation. In the case of *Prevotella intermedia* (p=0.025), a higher presence of bacteria was observed before preparation compared to the interval 15 minutes after preparation. In the case of *Tannerella forsythia* (p=0.016), the presence of the causative agent was significantly lower 15 minutes after preparation compared to the interval before preparation (p=0.031).

In group 2, consisting of subjects with the equigingival finish line position, there was a significant difference in the presence of *Aggregatibacter actinomycetemcomitans* during the observed period (p=0.023). This group exhibited a significant increase in the presence of the causative agent 72 hours after preparation compared to the interval 15 minutes after preparation (p=0.033).

Discussion

A potential diagnostic value of the gingival fluid is of particular importance for determining the degree of pathological changes in the periodontal tissue, especially if microorganisms from the subgingival dental plaque and markers indicating inflammation are detected in it[39]. It is quite certain that tooth preparation, especially with the subgingival finish line, causes mechanical trauma, a possible change in the oral flora, and consequent gingivitis. However, to date, no clinical study comparing different parameters of gingival inflammation has been conducted. Recent results of Jovanović et al[40] proved inflammatory clinical and cytomorphometric changes in the gingiva after tooth preparation in the observation period used in this study as well.

To obtain the most objective results, a rather homogeneous group of subjects (aged 30-40) was selected with an indication for the preparation of one canine in the upper jaw for PFM, with the subgingival and equigingival finish line. The absence of inflammation before tooth preparation was confirmed by clinical examination and the determination of gingival and bleeding indices, as well as by taking control samples of the gingival fluid[37]. It was assumed that tooth preparation changes the expression of MMP-9 and the distribution of periodontopathogens in the gingival crevicular fluid after tooth preparation. Moreover, these changes were found to depend on the location of the border between the prepared and unprepared tooth. Concentration changes were observed 15 minutes and 24 hours, i.e., 72 hours after the intervention. The gingival fluid represents an excellent diagnostic medium for biochemical and immunological findings in periodontal and peri-implant diseases[41,42].

MMP-9 is present in the sulcus and pocket epithelium, as well as in leukocytes and osteoclasts of the inflamed periodontal tissue[43,44]. It has been proven[45] that the concentration of MMP-9 in the gingival fluid increases with the severity of periodontal disease, i.e., that it decreases with its treatment[46]. An increase in the concentration of MMP-9 is closely related to the presence of specific periodontopathogens, such as *Tannerella forsythia* and *Treponema denticola*[47].

The results of this study show a change in the concentration of MMP-9 in the gingival fluid of the subjects after tooth preparation with the subgingival and equigingival chamfer finish line position. The MMP-9 values did not differ statistically significantly regarding the position of the finish line, either subgingival or equigingival.

The values of MMP-9 in both groups of subjects, determined in four observation intervals, showed a statistically significant difference in each of them. In the examined time, there was a statistically significant linear trend of increasing MMP-9 values and a significant quadratic trend with a decrease in MMP-9 values 15 minutes after preparation. In the further course of the examined time, the latter exhibited a significant increase, so that the values 72 hours after preparation were slightly higher than before the onset of the treatment. The obtained results indicated not only the existence but also the reversibility of the gingival inflammatory process.

The concentration of MMP-9 15 minutes after tooth preparation was significantly lower than the initial values of samples taken before tooth preparation in both groups of subjects. Reduced values can be explained by irrigation during preparation and by changing the real composition of the gingival fluid (by dilution) immediately after tooth preparation. Considering the described role of MMP-9 in the etiopathogenesis of gingival inflammation and periodontal diseases in general, it can be concluded that the expression of MMP-9 as a marker of inflammation was unexpected after such a short time from mechanical damage to the hard tooth tissue. On the other hand, 24 hours after the intervention, the concentration of MMP-9 increased compared to the control group, in both groups of subjects. This indicated the development of the inflammation of the gingiva, though of a reversible nature. With all precautions, the concentration of MMP-9 generally returned to the initial stage (control level) after the last observation interval, when the clinical and laboratory fabrication of planned prosthetic restorations continued. Vu and Werb[48] proved the involvement of MMPs in tissue remodeling by influencing cellular behavior, e.g., by inducing the migration of cells in normal growth and tissue remodeling, such as wound healing and angiogenesis.

The obtained values of MMP-9 concentrations were slightly higher in cases with the subgingival finish line, which indicates greater tissue damage and greater inflammatory potential of this type of preparation (Figure 1). Interestingly, the position of the finish line did not significantly affect the concentration of MMP-9 in any of the observation intervals. The study was based on the assumption that tooth preparation inside the gingival sulcus could lead to greater mechanical damage, which, along with a possible change in the microbial flora and exposure to subgingival anaerobes, would result in more intense inflammation of the gingiva. Certainly, all recommendations are in favor of the equigingival and supragingival completion of tooth preparation, primarily to prevent gingivitis, which was not confirmed by this study.

A large number of publications[45,49,50] have shown the expression and activity of MMPs in gingival inflammation, which has been proven by the analysis of gingival fluid and saliva samples. On the other hand, literature data[51-54] show a decrease in the concentration of MMPs after periodontal tissue healing, which is in accordance with the results obtained in this study. Lazar et al[55] proved that the expression of MMP-9 in the inflamed gingival tissue is not indicative of the

histological subtype of the disease (gingivitis and chronic periodontitis) but is related to the severity of the resulting damage.

Furthermore, literature data[56,57] show that MMP-9 is an essential modulator of host defense during the initial immunological phase. In turn, it can initiate a cascade involving the entire host defense mechanism, endothelial homeostasis leading to an increased risk of developing periodontal disease. This explains the increase in the concentration of MMP-9 in the gingival fluid one day after tooth preparation, by triggering the immunological resources of the gingiva. The resulting inflammation had a reversible character in our study. The obtained results are in positive correlation with the results of Söder et al[58]. They demonstrated that the concentration of MMP-9 is increased in the gingival fluid in the initial phase of periodontal disease and that it plays a key role in neoangiogenesis, which is related to the host's response to periodontal pathogens. Moreover, Gursoy et al[59] suggested that the MMP-9 expression is associated with periodontal tissue damage during the active stages of periodontitis.

Tooth preparation can change the composition of the gingival fluid, thus enabling the development of microorganisms that could have a harmful effect on the health of the gingiva and deeper periodontal tissues. In this regard, we compared the composition of the oral flora of the gingival sulcus before (control samples) and after preparation (experimental samples for observation intervals of 15 minutes, 24 h, and 72 h), with the subgingival and equigingival localization of the border of the prepared and unprepared part of the tooth.

In relation to earlier theories about the influence of the quantity of biofilm on the development of oral diseases, the current "specific plaque hypothesis" emphasizes the pathogenicity of only those biofilms in which specific microorganisms are present[60]. The presence of the examined microorganisms in the subgingival plaque results in the exacerbation of changes that already exist in the periodontium and the inflammatory reaction of the gingiva. The subgingival dental plaque includes the following: *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* (*Bacteroides forsythus*), *Campylobacter rectus*, *Eikenellacorrodens*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *Treponema denticola*[61]. In the exacerbation phase, the number of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Prevotella intermedia* increases, which explains

why they were chosen for detection using the PCR qualitative analysis in this study.

For any of the described microorganisms to cause disease, it is necessary to fulfill Koch's postulates adapted for periodontal diseases: greater presence in diseased lesions and absence in healthy places; cessation of disease after elimination and relapse after their reappearance; the host organism reacts to their presence with a cellular and humoral immune response and the production of antibodies; microorganisms possess virulence factors to overcome immune responses of the host[62]. Microorganism virulence factors refer to the capacity of microorganisms to invade the host's tissues through toxic molecules, bacteria, and other pathogens to obtain food or attach to cells. Thus, *Aggregatibacter actinomycetemcomitans* has the following virulence factors: leukotoxin, collagenase, endotoxin, epitheltoxin, and fibroblast inhibitory factor. Moreover, it is considered the main cause of aggressive periodontitis. *Porphyromonas gingivalis* contains collagenase, proteases, fibrolysin, endotoxin, and phospholipase A and is more common in chronic periodontitis[61-63].

The subjects in the study did not show signs of periodontal disease. Thus, the appearance of specific pathogenic components, which were included in the PCR analysis, was in favor of the exacerbation of the disease and the development of gingival inflammation.

In subjects with the equigingival finish line position, a statistically significantly higher presence of *Aggregatibacter actinomycetemcomitans* in the gingival fluid was found 72 hours after preparation, as well as *Prevotella intermedia* in samples obtained 15 minutes after preparation. The frequency differences in other observation intervals regarding *Tannerella forsythia* and *Porphyromonas gingivalis* in relation to the position of the finish line were not significant for any of the observed intervals.

The presence of periodontopathogens in both groups of subjects changed during the observed period. A significant difference was recorded in subjects with the subgingival finish line regarding Aggregatibacter actinomycetemcomitans, *Prevotella intermedia*, and *Tannerella forsythia*. *Aggregatibacter actinomycetemcomitans* exhibited an increase 24 hours after preparation compared to 15 minutes after preparation. In contrast, *Prevotella intermedia* was more present before the preparation compared to the interval 15 minutes after, which also applies to *Tannerella forsythia*, only with statistical significance.

With all the limitations related to the sample size, it can be concluded that the prevalence of periodontopathogens in the intervals after preparation is significantly lower for *Prevotella intermedia* and *Tannerella forsythia* in group 1, whereas *Aggregatibacter actinomycetemcomitans* is significantly more abundant after preparation in both groups. The presence of *Aggregatibacter actinomycetemcomitans* as the main cause for the development of periodontogenic diseases is an alarming fact that requires further clinical and laboratory research on the impact of established procedures such as PFM on gingival health.

It should be pointed out that the PCR technique in this study did not perform a quantitative, but only a qualitative analysis of microorganisms. Therefore, it is not possible to conclude to what extent their number changed. The determination of the number of bacteria would have greater scientific and clinical significance and will be the subject of future studies. On the other hand, the obtained results can be a clear guideline in the preliminary conclusions of the impact of tooth preparation on gingival inflammation and give clear recommendations on how to conduct further research to prevent the occurrence of periodontal diseases.

Many researchers[25,29,64-66] recommend the use of microbiological tests as additional diagnostic tools in identifying different forms of periodontal disease. Characterization of the subgingival microflora before, during, and after therapy is both a good diagnostic tool and a valid criterion for the effectiveness of therapy. Based on the composition of the subgingival film, the therapy of periodontal damage can be regulated. Literature data[25,31] suggest that the detection of periodontal pathogens above certain "critical" levels after active treatment indicates an increased risk of disease recurrence.

Other authors confirmed the involvement of the mentioned microorganisms in the etiopathogenesis of gingivitis and periodontitis. Salari and Kadkhoda[67] found a high concentration of anaerobic bacteria in patients with periodontal disease, especially in deep periodontal pockets. A study by Haffajee et al[31] showed a higher prevalence of *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, and *Selenomonasnoxia* in patients with periodontal tissue changes. Chaves et al[68] determined that the presence of *P. gingivalis* indicates the progression of periodontal disease and bone loss. Tran et al[69] found a significant correlation between the presence of *Tannerella forsythia* and periodontal ligament loss, compared to the absence of the bacteria. It is assumed that

Escherichia coli, Candida sp., Staphylococcus aureus, Pseudomonas aeruginosa, and *Bacteroides sp.*, as well as viruses, also play a role in the development of periodontal diseases[26].

On the other hand, these microorganisms can also be present in the healthy supporting tissue of the teeth. In their study, Watson et al[70] isolated *Porphyromonas gingivalis* in 80% of healthy children during and after puberty. Similar results were obtained by Predin et al[71] and de Cruz et al72 – 80%, i.e., Lau et al73 – 81.3% of healthy patients with *Porphyromonas gingivalis.*

The results[74] of the prevalence of periodontopathogenic microorganisms in dental plaque are often contradictory due to variations in the genotype distribution, owing to the ethnic and geographic origin of subjects, oral hygiene, differences in diet, consumption of alcohol, tobacco, etc.

A study by Söder et al[75] proved a significantly higher presence of *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*, Prevotella nigrescens, *Tannerella forsythia*, and *Treponema denticola* in periodontal pockets and higher expression of MMP-8 and MMP-9 in affected patients compared to controls. Yakob et al[76] showed that the presence of *Tannerella forsythia* or *Treponema denticola* increases the total level of MMP-8 in the gingival fluid. Further research[77,78] by the same authors showed that the presence of *Tannerella forsythia* and *T. denticola* is associated with increased levels of MMP-9 in most of the gingival fluid samples tested. *Treponema denticola* and *Porphyromonas gingivalis* can activate pro-MMP-8 and MMP-9. Further research into the cumulative effect of changes in the oral microflora in the gingival sulcus and the expression of MMPs and other pro-inflammatory factors would provide clearer explanations of the impact of tooth preparation as an established dental procedure on changes in the periodontal tissue.

Regardless of the small number of subjects, this study confirms that tooth preparation changes the composition of the gingival fluid and can cause inflammation and damage to the gingival tissue. The drawback of the clinical study is the relatively small number of subjects. On the other hand, the inclusion factors were extremely strict, which gives the obtained results greater scientific significance. They certainly provide a good basis for further research into the impact of tooth preparation on the inflammation of the gingiva, which, in addition to the therapeutic effects (choice of preparation technique, durabi-

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lity of prosthetic restoration), also has protective significance in terms of protecting periodontal tissues from possible iatrogenic damage.

Conclusions

The value of MMP-9 concentration in patients with the subgingival and equigingival finish line position changed significantly during the follow-up period in the study. It was determined that the value 15 minutes after preparation significantly decreased compared to the values before preparation, and that it increased significantly after 24 hours. The value 72 hours after preparation was near the initial value or slightly higher, which indicates the reversibility of the inflammatory process. Having monitored the presence of bacteria in the gingival cervical fluid, it was found that periodontopathogens do not have a uniform response to inflammation in the period after preparation.

Further studies will be directed towards a comparative analysis of various parameters of inflammation in patients after tooth preparation to provide clear therapeutic and prophylactic recommendations in order to increase the longevity of prosthetic restorations along with the protection of the periodontal tissue.

Ethics Approval

The Ethics Committee of the Faculty of Medicine reviewed and approved the study (Decision No.: 68/5-2019-3EO).

Informed Consent

Informed consent was obtained for the tooth preparation and taking gingival fluid content samples.

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Authors' Contributions

MJ, MK: conceptualization, study design, data collection, manuscript writing, data analysis, interpretation and revision of the work. NSĐ, AI: data analysis, interpretation and revision of the work. UT, NN: data analysis, data interpretation. LjK: conceptualization, study design, interpretation, revision of the work. NG, MI, RJ, AĐ, SS: critical revisions related to relevant intellectual content. JM: data analysis, interpretation, revision of the work. The authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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