

ASSESSMENT OF PERIODONTOPATHOGENS IN SUBGINGIVAL BIOFILM OF BANDED AND BONDED MOLARS IN EARLY PHASE OF FIXED ORTHODONTIC TREATMENT

KRISZTINA MÁRTHA¹, LILLA LŐRINCZI^{2*}, CRISTINA BICĂ³,
RÉKA GYERGYAY³, BLANKA PETCU⁴, LUMINIȚA LAZĂR⁵

¹Department of Orthodontics, Faculty of Dental Medicine,
University of Medicine and Pharmacy, Tirgu Mures

²Department of Microbiology, Faculty of General Medicine,
University of Medicine and Pharmacy, Tirgu Mures

³Department of Pediatric Dentistry, Faculty of Dental Medicine,
University of Medicine and Pharmacy Tirgu Mures

⁴Department of Preventive Dentistry, Faculty of Dental Medicine,
University of Medicine and Pharmacy, Tirgu Mures

⁵Department of Periodontology, Faculty of Dental Medicine,
University of Medicine and Pharmacy, Tirgu Mures

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To assess the prevalence and occurrence of eleven periodontopathogens in subgingival biofilm of banded and bonded molars during the first period of fixed orthodontic treatment. Subjects were selected from patients referred to orthodontic treatment and were divided in two groups: group A comprised fifteen patients (14.4±2.45 years of age) who received orthodontic bands on first permanent molars and group B of ten patients (15.7±1.87 years of age) with directly bonded tubes on the labial surface of the same teeth. Subgingival sample collection was performed before bands and tubes application and 4–7 weeks after attachment placement. DNA-strip technique was used to assess the presence of eleven putative periodontopathogens at each time point. *Fusobacterium nucleatum*, *Eikenella corrodens* and *Capnocytophaga* spp. were found in a large number of samples, other periodontopathogens were present in a smaller rate. The 4–7 weeks after attachment placement a slight increase of putative species was observed in both groups.

*Corresponding author; E-mail: lillalorinczi@yahoo.com

The presence of orthodontic tubes and bands influence the accumulation and composition of subgingival microbiota. Higher level of oral hygiene should be achieved before and during orthodontic treatment in order to prevent any side effects on periodontal tissues.

Keywords: periodontopathogens, banded and bonded molars, subgingival biofilm, DNA-strip technique

Introduction

Fixed orthodontic treatment is a commonly used method to treat different malocclusions. Orthodontic bands and different attachments can make plaque removal and optimal oral hygiene difficult and this can lead to plaque retention and subsequent gingival inflammation from mild to severe form, including false pocket appearance [1]. Gingivitis can appear early due to the changes of subgingival biofilm composition and inflammatory reactions will lead to gingival enlargement. The development of gingival hyperplasia occurs more frequently in the posterior region and affects mainly the interproximal sites [2]. Long-term effect of orthodontic fixed appliances on periodontal health can be considered as a main topic in orthodontic research, most of the retrospective studies did not find any effect of it upon later periodontal health in adolescent patients.

Orthodontic treatment plan sometimes includes the preparation of the anchorage and the most frequently selected anchorage teeth are the first permanent molars. Orthodontic bands need to be adapted and especially in partially erupted teeth they have to be placed subgingival. Direct mechanical irritation of gingival tissues, chemical irritation due to the cement used for banding and greater plaque retention were establish as the main causes of the inflammatory phenomenon in this region [3].

The use of direct bonded labial tubes simplifies practitioner's chair side work and mechanical plaque removal is easier for the patient. Comparing the plaque accumulation, the level of gingival inflammation and loss of attachment on bonded and banded molars, some studies revealed significantly greater plaque deposits and more severe forms of gingival inflammation on banded molars and gingival problems were found more severe three months after debonding compared with bonded molars [4, 5].

Supra- and subgingival plaque composition during orthodontic treatment was studied using different methods. Increase of black-pigmented *Bacteroides*, *Spirochaetes*, filaments, fusiforms and motile rods found by using qualitative microbiologic tests and specific culture methods demonstrated the presence of

Actinobacillus actinomycetemcomitans in subgingival flora of young orthodontic patients [6].

DNA-strip technology, beyond its' high sensitivity, it is a relatively cheap and quick method to isolate, amplify and detect DNA fragments even from relatively poor quality DNA material. The micro-Ident®*plusII* test is a qualitative *in vitro* test for the combined identification of periodontopathogenic bacterial species from subgingival plaque sample, it can detect the following species: *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), *Treponema denticola*, *Parvimonas micra* (formerly *Micromonas micros*, *Peptostreptococcus micros*), *Fusobacterium nucleatum/periodonticum*, *Campylobacter rectus*, *Eubacterium nodatum*, *Eikenella corrodens* and *Capnocytophaga* spp. (*C. gingivalis*, *C. ochracea*, *C. sputigena*).

Therefore, the aim of this study was to assess the composition of subgingival biofilm and to evaluate the presence of the above-mentioned eleven putative periodontopathogens and to compare plaque composition of bonded versus banded molars after 4–7 weeks of attachment wearing.

Materials and Methods

After research protocol was established, it was approved by the Ethical Committee of Scientific Research of the University of Medicine and Pharmacy Tîrgu Mures, decision nr. 117/21.11.2013. Twenty-five consecutive subjects, 14 girls and 11 boys, aged 11–17 years were selected from patients referred for orthodontic treatment at the Orthodontic Department of Faculty of Dentistry, University of Medicine and Pharmacy Tîrgu Mureş, Romania and were divided in two groups: group A comprised fifteen patients (14.4±2.45 years of age) who received orthodontic bands on first permanent molars and group B of ten patients (15.7±1.87 years of age) with directly bonded tubes on the labial surface of the same teeth. For both groups the selection was made by the following criteria: (1) good general condition, with no signs of systemic or local disease which could affect gingival response, (2) good oral hygiene practice prior to appliance placement, which was maintained between the two examination times, (3) no use of anchorage reinforcement auxiliary appliance which could interfere with mechanical plaque removal, (4) good periodontal condition with low plaque index (PI<2) and gingival index (GI≤1) before the commencement of the treatment and at T2 sample collection as well, (5) no use of any antibiotic treatment three month before attachment placement, (6) the same type of bands and bonds

(Dentsply GAC International, USA), the same bonding material (Transbond™ XT, 3M Unitek, USA) and the same band adhesive (Transbond™ Plus, 3M Unitek, USA) were used.

Sample collection

Informed consent was obtained from selected subjects and their legal representative before first sample collection after all the information was provided about the study. Before both sample collections sites were isolated with cotton rolls and were air-dried. Supragingival plaque was removed with a sterile point and biofilm collection was performed using sterile paper points (Micro-Ident Sampling Set, Hain Lifescience GmbH, Germany). Samples were collected in five sites for each permanent first molar: disto-oral, disto-labial, mesio-oral, mesio-labial and centro-labial points. Paper points were transferred into a transporting screw cap tube, transported to the Department of Microbiology within 72 hours.

Microflora

DNA isolation was performed using DNA-strip technique (QIAamp® DNA Mini Kit, Qiagen) after the following protocol: (1) 200 µl of ATL buffer was added to each tube and vortexed for 30 seconds, (2) mixed with 20 µl Proteinase K solution and incubation at 72° for 10 minutes, (3) adding 200 µl AL buffer, vortexed for 15 sec and incubate for 5 min at 95°, (4) adding 200 µl ethanol (96–100%), vortex 15 s, (5) spinning of 700 µl of solution for 1 minute at approx. 8000 rpm, (6) repeat spinning after adding 500 µl of buffer AW1, (7) spinning for 3 minutes at maximum speed after adding 500 µl of buffer AW2, (8) 72° C pre-warmed AE buffer is added for DNA elution, incubate for 1 min at room temperature and spin down for 1 min at 8000 rpm.

Two separate amplification reactions were used for each sample, using the optimized polymerase and primers included in the Amplification Mixes A1 (AM-A1 for DNA from *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia* and *T. denticola*), A2 (AM-A2 for DNA from *P. micra*, *F. nucleatum/periodonticum*, *C. rectus*, *E. nodatum*, *E. corrodens* and *Capnocytophaga* spp.) and B (AM-B – an amplification mix which contains buffer, salts and dye). Twenty-five µl of amplification 1 (5 µl AM-A1, 17.5 µl AM-B and 2.5 µl DNA solution) and amplification 2 (5 µl AM-A2, 17.5 µl AM-B and 2.5 µl DNA solution) solutions were prepared for each sample. Amplification was made

for twelve samples in one procedure, so after preparing the master mixes (1 and 2) control samples (to detect possible contamination, these samples contain water instead of DNA solution) were included, too. Amplification profile include one cycle (5 min, 95°), ten cycles (30 sec at 95° and 2 min at 58°) 20 cycles (25 sec at 95°, 40 sec at 53° and 40 sec at 70°) and one cycle (8 min at 70°).

Manual hybridization was performed. In each well used 20 µl of denaturation solution was dispensed and 20 µl of amplicon 1 and 20 µl of amplicon 2 was added and incubated at room temperature for 5 minutes. Once the hybridization buffer was added, a strip in each well was placed and 30 minutes incubation at room temperature in shaking water bath followed. Hybridization buffer was completely aspirated, 1 ml of stringent wash solution was added to each strip and incubated for 15 minutes at 45° in shaking water bath. After the removal of the stringent wash solution, each strip was washed once with 1 ml rinse solution for 1 minute on shaking platform. One ml of diluted conjugate solution was added to each strip, followed by incubation for 30 minutes on shaking platform. Conjugate solution was totally removed, strips were washed twice for 1 minute with 1 ml of rinse solution and once for 1 minute with approx. 1 ml of distilled water on shaking platform. One ml of diluted substrate was added to each strip and 3–20 minutes incubation protected from light without shaking followed. As soon as bands became clearly visible, they were rinsed with distilled water and dried between two layers of absorbent paper.

Strips were evaluated and interpreted using the evaluation sheet provided with the kit. Validation of the quality control of the test was performed by the assessment on the strips of the following control zones: conjugate control (CC) zone, the binding of the conjugate and the chromogenic reaction; hybridization control zone (HC) to check the successful hybridization and amplification control zones (AC1 and AC2) to check the successful amplification reactions. If test was performed correctly, the control amplifications AC1 and AC2 bound to the respective amplification control. Valid negative results were considered those, where only the CC, HC, AC1 and AC2 bands were developed.

Sample collection was performed at twice: (T1) right before tube or band placement and (T2) after attachment placement. Between the two appointments subjects were encouraged for proper use of manual toothbrush and interdental cleaning devices and no professional cleaning was performed.

Data analysis

The data were recorded and entered into Microsoft excel sheet and analysed. Associations between the prevalence of each periodontopathogen from T1

Table 1. Frequency of periodontopathogens in subgingival biofilm at twice of orthodontic treatment before bonding (T1) and 4–7 weeks after bracket placement (T2) on banded (group A) and bonded (group B) molars

| Periodontopathogen | Group A (n = 15) | | Group B (n = 10) | |
|---|------------------|-----------------|------------------|----------------|
| | T1 | T2 | T1 | T2 |
| <i>Aggregatibacter actinomycetemcomitans</i> (A.a.) | n = 0 | n = 0 | n = 2 (20.00%) | n = 2 (20.00%) |
| <i>Porphyromonas gingivalis</i> (P.g.) | n = 2 (13.33%) | n = 3 (20.00%) | n = 0 | n = 0 |
| <i>Prevotella intermedia</i> (P.i.) | n = 3 (20.00%) | n = 3 (20.00%) | n = 1 (10.00%) | n = 1 (10.00%) |
| <i>Tanarella forsythia</i> (T.f.) | n = 5 (33.33%) | n = 8 (53.33%) | n = 3 (30.00%) | n = 3 (30.00%) |
| <i>Treponema denticola</i> (T.d.) | n = 3 (20.00%) | n = 7 (46.66%) | n = 4 (40.00%) | n = 5 (50.00%) |
| <i>Parvimonas micra</i> (P.m.) | n = 4 (26.66%) | n = 8 (53.33%) | n = 1 (10.00%) | n = 3 (30.00%) |
| <i>Fusobacterium nucleatum</i> (F.n.) | n = 13 (86.66%) | n = 15 (100%) | n = 10 (100%) | n = 10 (100%) |
| <i>Campylobacter rectus</i> (C.r.) | n = 4 (26.66%) | n = 5 (33.33%) | n = 3 (30.00%) | n = 5 (50.00%) |
| <i>Eubacterium nodatum</i> (E.n.) | n = 0 | n = 2 (13.33%) | n = 0 | n = 0 |
| <i>Eikenella corrodens</i> (E.c.) | n = 10 (66.66%) | n = 13 (86.66%) | n = 9 (90.00%) | n = 9 (90.00%) |
| <i>Capnocytophaga</i> spp. (C.sp.) | n = 13 (86.66%) | n = 14 (93.33%) | n = 6 (60.00%) | n = 9 (90.00%) |
| Overall positive sites frequency | 34.54% | 47.27% | 35.45% | 42.72% |

to T2 sample in both groups were analysed using Fischer's exact test (GraphPad InStat). The same test was used to compare the overall subgingival biofilm composition for both (A and B) groups at T1 and T2 sample collection ($p < 0.05$ was considered statistically significant).

Results

Subgingival biofilm composition changed after one month of band or directly bonded attachment placement. *Eubacterium nodatum* was not identified in neither group A, nor group B subjects at T1 sampling, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* was totally missing from group B T1 samples (Table I).

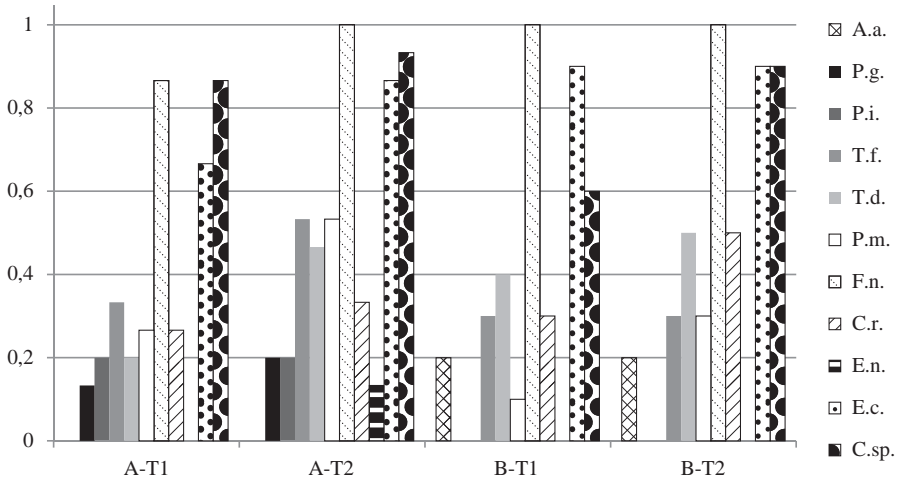


Figure 1. Comparison of the prevalence of the studied periodontopathogens in both groups, at T1 and T2 sample collection

Table II. Statistic relationship between positive site frequencies in both groups

| Periodontopathogen | Group A P value – T1 vs T2 ^a | Group B P value – T1 vs T2 ^a |
|----------------------------------|--|--|
| <i>Tanerella forsythia</i> | 0.46 | – |
| <i>Treponema denticola</i> | 0.24 | – |
| <i>Parvimonas micra</i> | 0.26 | – |
| <i>Fusobacterium nodatum</i> | 0.48 | – |
| <i>Eubacterium nodatum</i> | 0.48 | – |
| <i>Eikenella corrodens</i> | 0.38 | – |
| <i>Capnocytophaga</i> spp. | – | 0.30 |
| Overall positive sites frequency | 0.025* | 0.333 |

^aFischer’s exact test (GraphPad InStat), *P<.05

T1 subgingival biofilm samples showed an elevated number of those cases, where *Fusobacterium nodatum*, *Eikenelle corrodens* and *Capnocytophaga* spp. in both groups. After one month of orthodontic attachment placement *Eikenella corrodens*, *Parvimonas micra*, *Treponema denticola* and *Tanerella forsythia* (A group) and *Capnocytophaga* spp. (B group) showed greater prevalence (Fig. 1).

Analysing the different types of putative species, no statistical significances were found between the prevalence of one bacteria at the two sample collection time ($0.26 < p < 0.48$). Comparing the overall changes of biofilm composition separately for A and B groups between T1 and T2 samples, increase of periodontopathogens percentage was statistically significant for the A group ($p = 0.025$) and was statistically insignificant for group B ($p = 0.333$) (Table II).

As results showed, plaque composition changes both in occurrence and in presence of putative periodontopathogens in the first stage of orthodontic treatment. No significant statistical differences were found among the presence of the above-mentioned species.

Discussions

Gingivitis is the most common periodontal diseases among children and adolescents with a prevalence of 80% at 11–13 years of age and a peak of severity around the onset of puberty. The composition of subgingival biofilm is age-dependent, anaerobic genera show a different percentage in it. Changes in biofilm composition during adolescent period of life can be explained with increase plaque accumulation due to improper oral hygiene. As soon as orthodontic appliances are bonded, plaque removal becomes more difficult to accomplish and moderate gingivitis can be detected in the early stage of this kind of treatment [7].

Several studies revealed that some of the periodontopathogenic bacteria can be present in gingival crevicular fluid of children with healthy periodontium [8–11]. Subgingival plaque composition of gingivitis free children presented almost the same percentage of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* and no statistical differences were found between the level of the above-mentioned species compared with children with gingival inflammation [8, 10, 12].

Analysing the role of different types of periodontopathogenic species, Socransky et al. found out, that four factors have to come together in order to maintain periodontal processes: a host with inadequate oral hygiene, a defective immune system, an increased number of pathogenic bacteria and a reduced number of “nonpathogenic” species of microorganisms. The same author grouped and colour-labelled these species and revealed that pathogens from red group are considered late colonizers with high pathogenicity (*P. gingivalis*, *T. forsythensis* and *Treponema denticola*) [13].

Analysing the initial biofilm composition (T1 sample in both groups) our results showed, that most of the periodontopathogen bacteria, we have been following, were present in the subgingival plaque of our subjects. Two species – *Fu-*

sobacterium nucleatum (92%) and *Eikenella corrodens* (76%) – were present in over three-quarter of the samples. These two species were grouped by Socransky in the second colonizers group and were found species with low and moderate pathogenicity. Highly pathogen species were missing from both samples (*Porphyromonas gingivalis* in B group) or were found in few cases (*Tanerella forsythia* and *Treponema denticola* in both groups). Overall sample composition showed a very pronounced diversity, even though moderately pathogen, second colonizers (*Aggregatibacter actinomycetemcomitans* and *Eubacterium nodatum*) were not found in subgingival plaque before the commencement of fixed orthodontic treatment.

Assessing the evolution of the different types of periodontopathogens in the two studied groups, it becomes relevant, that in some cases a slight increase of percentage can be observed from T1 sample to T2 sample in both groups. The same results were found by several studies, this might show in only a few days second colonizers appear, as *P. gingivalis*, *T. forsythensis*, and *F. nucleatum* will become present in a higher rate [14]. *T. forsythia*, *C. rectus* and *P. nigrescens* significantly increased after placement of orthodontic appliances, meanwhile for the other periodontopathogen species the frequency tended to increase but no statistically significant difference was noted [15].

Our results confirmed that subjects from group A, where orthodontic bands were used on anchorage teeth, and the prevalence of putative periodontopathogens increased statistically significant in early phase of fixed orthodontic therapy. Some clinical studies confirmed the difference between periodontal response when bands or directly bonded tubes are used [2, 16]. More inflammation and plaque retention on bands can explain the following gingival reaction in these cases.

Conclusions

The findings of the present study show that there is a difference in composition of subgingival biofilm of bonded and banded molars, even though an increase of periodontopathogens can be observed in both situations in early phase of orthodontic treatment. When bands are used, the difference in plaque composition alters significantly in the first 4–7 weeks of fixed orthodontic treatment. Once orthodontic attachments are bonded, composition of subgingival biofilm shows a greater diversity but statistically this change was significant only when bands were used on anchorage teeth. Further studies regarding the behaviour of these species during later phases of treatment and after appliance removal are

required. Good oral hygiene before and during orthodontic treatment is the only way to avoid periodontal damage which may be caused by orthodontic attachments.

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Conflict of interest

No potential conflicts of interest are disclosed.

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