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„Evaluation of clinical indices, microbiological and matrix metalloproteinase-8 levels in subgingival biofilm of patients with fixed appliance, before and during orthodontic treatment“

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II Abreviation

α	Alpha
%	Percent
±	Plus/Minus
>	Comparison symbol „greater than“
≥	Comparison symbol „greater than or equal to“
<	Comparison symbol „less than“
≤	Comparison symbol „less than or equal to“
ml:	Milliliters (Unit)
mm:	Millimeters (Unit)
ng:	Nanogram (Unit)
µl:	Microliter (Unit)
Aa:	*Aggregatibacter actinomycetemcomitans*
AIDS:	Acquired immune deficiency syndrome
AL:	Attachment loss
aMMP-8:	Active matrix metalloproteinase-8
ANUG:	Acute necrosing ulcerative gingivitis
BOP:	Bleeding on probing
CAL:	Clinical attachment level
Cr:	*Campylobacter rectus*
Cs:	*Capnocytophaga spp.*
DNA:	Deoxyribonucleic acid
Ec:	*Eikenella corrodens*
ELISA:	Enzyme-linked immunosorbent assay
En:	*Eubacterium nodatum*
Fn:	*Fusobacterium nucleatum*
Fs:	*Fusobacterium spp*
GCF:	Gingiva crevicular fluid
GI:	Gingival index
HIV:	Human immunodeficiency virus
IQD:	Interquartile distance

LJP:	*Localized juvenile periodontitis*
LOJ:	Lower jaw
Max.:	Maximum
MBA:	Multibracket appliance
Min.:	Minimum
MMP:	Matrix metalloproteinase
MMP-8:	Matrix metalloproteinase-8
MMPs:	Matrix metalloproteinases
mQHI:	modified Quigley-Hein index
MV:	Mean value
OHI:	Oral hygiene instructions
PCR:	Polymerase chain reaction
PD:	Periodontal disease
Pg:	*Porphyromonas gingivalis*
PI:	Plaque index
Pi:	*Prevotella intermedia*
Pm:	*Parvimonas micra*
Pn:	*Prevotella nigrescens*
PP:	Periodontopathogens
PPD:	Pocket probing depth
QHI:	Quigley-Hein index
RNA:	Ribonucleic acid
SD:	Standard deviation
T:	Time point
Tf:	*Tannerella forsythia*
TIMPs:	Tissue inhibitors of metalloproteinases
Td:	*Treponema denticola*
UPJ:	Upper jaw

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1. Introduction

Dental and skeletal malocclusions can have a negative impact on quality of life by interfering with patient´s aesthetics, social interaction, and psychological well-being (1-5). Moreover, it can affect functions of the stomatognathic system such as breathing, chewing, and swallowing (6-8). Due to these reasons, the malocclusion should be treated.

The orthodontic treatment with fixed appliance is a widely used method for the treatment of malocclusions. However, the components of this appliance such as brackets, arches, ligaments, and tubes make oral hygiene difficult, affecting oral health by the increased accumulation of biofilm around the retentive structures (9-11).

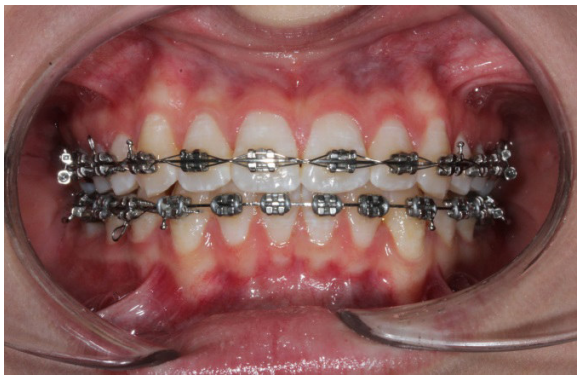


Figure 1: Multibracket appliance in situ; Photo: P. Ferrari Peron.

Thus, the high number of retention surfaces for biofilm together with poor oral hygiene can contribute to the development of dental caries (12-14), gingivitis (15-17) and periodontal attachment loss (18-21). Enamel demineralization and the associated development of white spot lesions (22-25), and gingival inflammation (18, 19, 26) are the most common negative effects associated with the use of this appliance.

Furthermore, the presence of a multibracket appliance (MBA) can change the composition of dental biofilm, leading to pathogenic bacterial colonization (26-30). A significant change in subgingival biofilm composition is observed after MBA with a

prominent increase in the concentration of periodontopathogens such as *Aggregatibacter actinomycetemcomitans* (30-32) *Porphyromonas gingivalis* (31-34) *Tannerella forsythia* (34), *Prevotella intermedia* (31, 32). Moreover, these pathogenic bacteria are responsible for the development of gingivitis and periodontal tissue destruction (31-34).

Increased levels of matrix metalloproteinases (MMPs) found in gingival crevicular fluid, salivary fluid and gingival tissues have been associated with periodontal disease (35-37). MMPs are known to play a role in periodontal tissue degradation in periodontitis and also in periodontal ligament remodeling during orthodontic tooth movement (37-41).

Since MBA treatment can negatively affect patients' oral health, the present study aimed to evaluate clinical and microbiological aspects of MBA patients at different time points up to 1 year after MBA placement. For these purposes, a gingival index and a plaque index were evaluated as well as the levels of active matrix metalloproteinase-8 (aMMP-8) and 11 periodontopathogens in subgingival biofilm.

The following hypotheses were formulated:

a)

H0: There is no correlation between gingival index values before and during treatment.

H1: There is a correlation between gingival index values before and during treatment.

b)

H0: There is no correlation between dental plaque index values before and during treatment.

H1: There is a correlation between dental plaque index values before and during treatment.

c)

H0: There is no correlation between the pro inflammatory biomarker MMP-8 in the gingival crevicular fluid before and during treatment.

H1: There is a correlation between the pro inflammatory biomarker MMP-8 in the gingival crevicular fluid before and during treatment.

d)

H0: There is no correlation between periodontopathogens before and during treatment.

H1: There is a correlation between periodontopathogens before and during treatment.

2. Literature Review

Caries, periodontal diseases, lip and mouth cancer are the most prevalent and serious oral diseases that affect the worldwide population (42).

Orthodontic treatments often last for many years until the ideal result is obtained. Several studies have shown that MBA use can lead to undesired side effects, such as periodontal diseases (10, 17-19) and initial lesions of caries, so-called white spot lesions (22-25). The development of periodontal diseases and initial caries lesions occurs due to plaque accumulation on dental surface. When hygiene is inadequate for a long period of time, it may have a negative effect on the gingiva (16, 20, 21) and tooth enamel (43, 44).

2.1 Periodontal Disease

Periodontal disease (PD) is the general denomination used to describe the inflammatory response of the gingiva and surrounding supporting tissues to bacterial biofilm (plaque) on dental surface (45). It is an infectious disease, multifactorial with complex pathogenesis that can lead to tooth-supporting tissues destruction and to tooth loss (46-50).

During almost the last two decades, periodontal diseases were categorized according to the International Workshop for a Classification of Periodontal Disease from 1999. Based on this classification, the PD was divided into: Gingival diseases (subgroups: Dental plaque induced gingival lesions and non-plaque induced lesions) and Periodontitis (subgroups: Chronic Periodontitis, Aggressive Periodontitis, Periodontitis as a manifestation of systemic diseases, Necrotizing Periodontal Diseases, Abscesses of the Periodontium, Periodontal-Endodontic Lesions, Development or Acquired Deformities and Conditions) (51).

There is a new classification of PD since 2017. The article published by Caton et al. summarized the meeting of the World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions occurred in 2017. In this meeting it was presented an update classification of periodontal diseases from 1999 and a new classification of peri-implant diseases. According to this new classification, the periodontal and peri-implantat diseases are divided into 2 groups: Periodontal Diseases and Conditions and Peri-Implant Diseases and Conditions and each group subdivided into smaller categories (52). Relevant for the orthodontic treatment with MBA are the following periodontal diseases: gingival and periodontal heathy, gingival diseases, periodontitis and necrotizing periodontal diseases.

Based on the new classification, gingivitis is now denominated as „Gingivitis Non-Dental Biofilm-Induced" and "Gingivitis Dental Biofilm-Induced" (52). The first one is not caused by plaque and occurs due to manifestations of systemic conditions like genetic/endocrine disorders, inflammatory and immune conditions, traumatic lesions, neoplasms and gingival pigmentation (53). The second one is an inflammatory reaction of the marginal gingiva to dental biofilm. The inflammation affected only the gingiva and does not expand to the periodontal supporting tissues (periodontal ligament, cement and alveolar bone) (54). Gingivitis is clinically characterized by redness, swelling, bleeding on probe, exudation, and ulceration, without attachment loss or bone resorption. It is total reversible after reduction levels of plaque (16, 21, 45, 54).

Periodontitis is reclassified as: "Periodontitis", "Necrotizing Periodontal Diseases" and "Periodontitis as a Manifestation of systemic diseases" (52). Periodontitis is a disease with multifactorial etiology, chronic, and irreversible. Clinically is characterized by attachment loss (AL), periodontal pocket, gingival bleeding. Bone alveolar loss can be assessed using radiography. Necrotizing periodontal diseases are clinically manifested by papilla necrosis, gingival bleeding and pain. To this group belong *Necrotizing gingivitis, Necrotizing periodontitis* and *Necrotizing stomatitis* (52, 55, 56).

2.2 Periodontopathogens

The relationship between periodontal disease and biofilm is already well established and the actual literature reports the occurrence of more than 700 bacterial species in the oral environment (57-60). Although most of the bacteria existing in the microbiota oral are commensal and coexists in harmony with the host, there is a number of them that are opportunist and able to cause PD (61-65) or caries (65-67). Some of them can cause systematic diseases (57, 68), as well as bacterial endocarditis (69, 70), osteomyelitis (71), among others.

The studies conducted by Paster et al. (57) and by Wade (61) investigated the bacterial flora in human subgingival plaque and could identify more than 450 species. These species were categorized in 15 different phyla and about 96% of the bacteria found in oral microbiome belong to 6 different phyla: *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Firmicutes*, and *Spirochaetes*. Figure 2 shows these phyla with the most common species found in human subgingival biofilm.

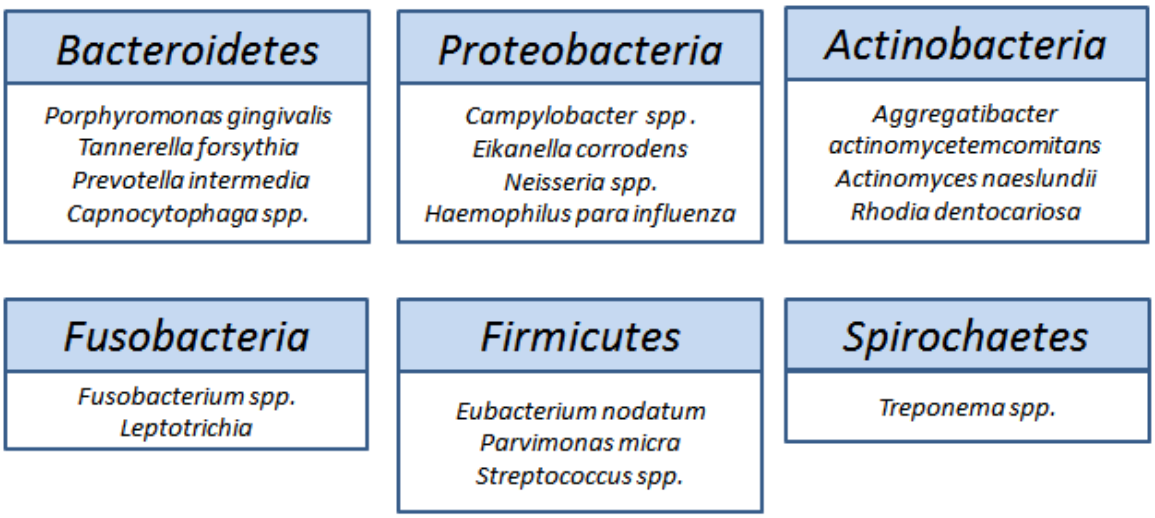


Figure 2: Bacterial diversity identified in human subgingival plaque according to Paster et al. (57) and Wade (61); edited: P. Ferrari Peron.

Socransky et al. (72) investigated the relationship among the bacteria found in subgingival plaque samples. A total of 185 subjects (age 20-87) were selected for this study. Twenty-five of them had no signs of gingivitis, periodontitis or AL and 160 had signals of PD with clinical AL. Based on their findings, the bacteria were grouped into 5 complexes with closely related species. The first or “red complex” is formed by 3 tightly related species: *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola.* The second complex or “orange complex” is constituted of *Fusobacterium spp*, *Prevotella intermedia*, *Prevotella nigrescens* and *Parvimonas micros. Eubacterium nodatum*, *Campylobacter spp and Streptococcus constellatus* are species highly associated with this complex and are also part of this complex. The third complex, called “yellow complex” is formed by 6 *Streptococcus species: S. sp.*, *S. sanguis*, *S. oralis*, *S. mitis*, *S. gordonii* and, *S.intermedius*. The fourth complex or “green complex” includes *Capnocytophaga spp*, *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* serotype a. The last complex is also known as “purple complex”, consisted of *Veillonella parvula* and *Actinomyces odontolyticus. Actinomyces naeslundi*. *Aggregatibacter actinomycetemcomitans serotype b.* and *Selenomas noxia* could not be aggregated in any of the complex. Figure 3 illustrates the microbial subgingival complex.

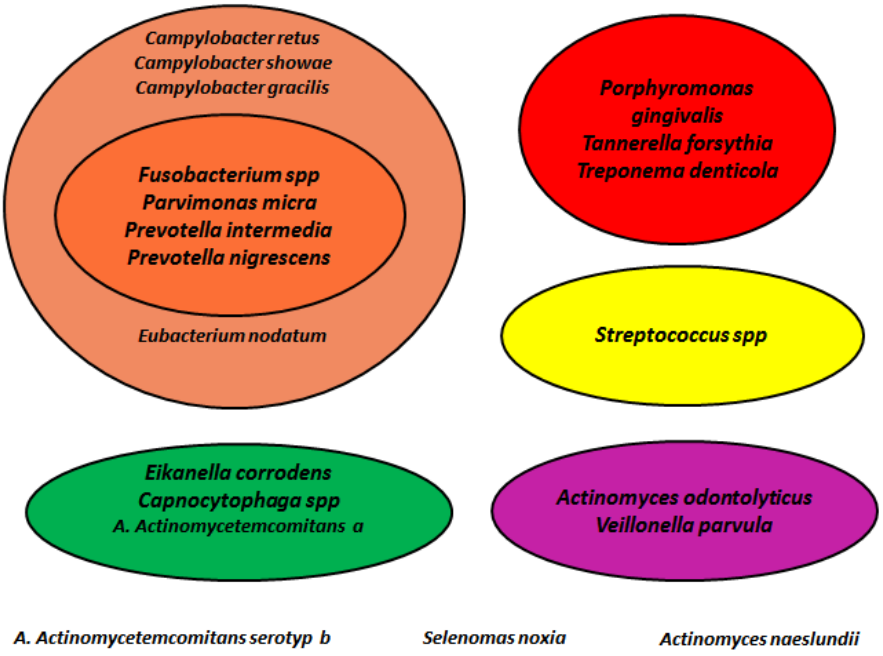


Figure 3: Microbial subgingival complex according to Socransky et al. (72); edited: P. Ferrari Peron.

The study performed by Ximénez-Fyvie and co-authors (73) compared and described the microbial composition of the supra and subgingival plaque samples of 23 patients (mean age: 51±14 years) with periodontitis. Forty different species were investigated, and results showed that all of them were detected in both supra- and subgingival plaque. Bacteria from “red” and “orange” complexes were the most prevalent in subgingival plaque samples. While in supragingival plaque, bacteria from “green” and “purple” complexes were the most predominant.

2.2.1 *Aggregatibacter actinomycetemcomitans*

Agreggatibacter actinomycetemcomitans (*Aa*) is a facultative gram-negative, anaerobic rod and has been long associated with destructive periodontitis in adults (49, 74-76). *Aa* had also been reported in the literature as the etiologic agent of local aggressive periodontitis (*localized juvenile periodontitis - LJP*). Stoks in 1976 was one of the first investigators who found *Aa* in the subgingival microbiota in sites of *LJP* (74, 77, 78).

A study conducted by Zambon (79) also found incresead levels of *Aa* in individuals affected by this disease. *LJP* was considered to occur among young adolescents and its classical localization was limited to the first molars and incisors; the first permanent teeth that erupt in the mouth around 6 years old. However, small amounts of *Aa* can also be found in healthy individuals as part of the oral cavity commensal flora (62, 76, 80).

2.2.2 Red complex

The bacteria *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*) and *Treponema denticola* (*Td*) comprise the red complex and are intimately associated with destructive periodontitis (81, 82) and also with endodontic infections (83).

Pg is a black-pigmented, gram-negative anaerobic rod, tissue invasive and is considered as the main pathogen of destructive periodontal disease in adults. This specie also occurs with high frequency in rapidly progressive periodontitis, pre puberty periodontitis, generalized juvenile periodontitis and tobacco-associated periodontitis (45, 57, 76, 84).

Tf earlier referred as *Bacteroides forsythus* (85) is an anaerobic gram-negative specie and is also considered an etiologic agent of chronic periodontitis. It is often reported in the presence of *Pg* and *Td* and is associated with gingivitis, chronic and aggressive periodontitis (72, 76).

Td is the major periodontal pathogen found in acute necrotizing ulcerative gingivitis (ANUG) (45, 57, 62, 76). It has been demonstrated in adult periodontitis and by patients with acquired immune deficiency syndrome (AIDS) (62, 86). However, this periodontal pathogen has also been detected in healthy patients (57, 62).

2.2.3 Orange complex

Prevotella intermedia (*Pi*) together with *Fusobacterium spp* (*Fs*) and *Parvimonas micra* (*Pm*) form the orange complex (72, 73, 82). *Pi* is a black-pigmented, anaerobic, gram-negative bacillus, and like *Td* is also associated with ANUG. *Pi* was also found in patients with gingivitis, adult periodontitis, refractory periodontitis and tobacco-associated periodontitis (62, 76).

The members of phyla *Fusobacteria* include bacteria of the genera *Fusobacterium* and *Leptotrichia. Fusobacterium nucleatum* (*Fn*) or *Fusobacterium naviforme* is the most common pathogen found in sub- and supra gingival biofilm. It is one of the several bacteria detected in ANUG, chronic periodontitis and refractory periodontitis. *Fs* is known as an early colonizer considered as a bridge bacterium that favors the colonization of other bacteria, such as from the red complex. *Fs* is also detected in healthy patients (46, 62, 73, 81).

Pm is one of the gram-positive anaerobic cocci that inhabit the subgingival microflora. *Pm* is increased in cases of periodontal diseases but it can also be found in healthy patients. The high concentration of *Pm* is considered a risk factor for aggressive generalized periodontitis (46, 61, 76).

2.2.3.1 Bacteria associated to orange complex

Campylobacter rectus (*Cr*) and *Eubacterium nodatum* (*En*) are intimately associated with the orange complex (72). *Cr* is a gram-negative, anaerobic, small, mobile vibrio and was found in higher number and more often in individuals with active lesions of destructive periodontitis (44, 47, 84). *Cr* is, like the most already mentioned bacteria, an opportunistic pathogen that develops and lives in situations of immunodeficiency. Studies have demonstrated a high prevalence of *Cr* in patients with periodontitis and AIDS. It represents 20% of the total bacteria species found in the subgingival flora of these patients (86, 87).

En belongs to the group of gram-positive anaerobic bacilli and is considered a microbiological indicator of PD (61, 88). It has been demonstrated a strong association between *En* and *Td* with periodontitis (89).

2.2.4 Green complex

This complex is constituted by the bacteria *Eikenella corrodens (Ec)* and *Capnocytophaga spp (Cs)* (72). *Ec* is known as a gram-negative, regular and small rod. This specie has been observed in patients with osteomyelitis (76) and often in an individual with more frequent sites of periodontal destruction than in healthy individuals. *Ec* has also been reported in association with *Aa* in some lesions of *LJP* (90).

Cs is considered as early colonizers and is more frequently found in the supragingival biofilm (57). Some studies have shown a significantly higher prevalence of *Cs* in individuals with periodontitis and *diabetes mellitus* than in individuals with periodontitis but without *diabetes mellitus* (91, 92).

2.3 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are key enzymes in extracellular matrix degradation during organogenesis, growth, and tissue development. They are synthesized by different types of mesenchymal cells, hematopoietic cells, including monocytes and macrophages, keratinocytes, endothelial cells and also by some tumor cells. MMPs are calcium- and zinc-dependent endopeptidases and are secreted as proenzyme (latent form), which requires extracellular activation (93, 94). In adults, the expression and activity of MMPs are usually relatively low, but increases significantly in several pathological conditions that can lead to undesirable tissue degradation, such as inflammatory diseases, tumor growth, and metastasis (95, 96).

MMPs activation is regulated by changes in the balance between the expression and synthesis of MMPs and their principal endogenous inhibitors: tissue inhibitors of metalloproteinases (TIMPs). In heathy conditions, there is a balance between MMPs and TIMPs. In PD this balance is shifted towards greater MMPs activity, which leads to periodontal tissue destruction (96, 97).

There are nearly 30 different MMPs and they can be categorized into four major groups: collagenases (MMP-1/collagenase-1, MMP-8/collagenase-2, MMP-13/collagenase-3), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP-26), membrane-bound MMPs (MMP-14, -15, -16, -17, -24, -25) and others (93-95, 98).

2.3.1 Matrix Metalloproteinase-8

Matrix metalloproteinase-8 (MMP-8) is the most evident collagenase found in the gingival crevicular fluid (GCF) and salivary fluid of periodontal diseases patients. It is associated with connective tissue degeneration and progression of PD. When active, this enzyme has the capability to decompose types I and III collagens, which are important for the maintenance of dental support tissues (37, 99-102).

The virulence factors of the periodontopathogens (PP) of the supra and subgingival plaque cause an immune-inflammatory reaction by the host. Neutrophils (*"polymorphonuclear leukocytes"*), the first cells in the line of defense against bacteria present in the biofilm, release MMP-8, which is subsequently activated (aMMP-8). In this activated form, aMMP-8 decomposes periodontal tissue collagen and is also associated with alveolar bone destruction. Thus, aMMP-8 is an organism enzyme itself, responsible for the tooth-supporting tissues destruction in PD (50, 103).

Several studies have demonstrated that aMMP-8 values are significantly more elevated in patients with PD than in healthy patients (102, 104-107). For this reason, aMMP-8 has been used, in addition to PP, for PD diagnosis (105, 108, 109). Some studies have also demonstrated that the aMMP-8 level can be used as a severity PD indicator in pregnant patients (110), patients with *diabetes mellitus* (111), coronary heart disease (112, 113), rheumatoid arthritis (114, 115), and peri-implantitis (96, 116).

Traditional periodontal diagnostic procedures during clinical investigations such as clinical attachment level (CAL), bleeding on probing (BOP), gingival index (GI), plaque index (PI) or x-rays are only visible after the presence of inflammation or biofilm formation, or even after the presence of partially irreversible periodontal damage. Interestingly, aMMP-8 concentration assessment in GCF or salivary fluid allows much earlier, non-invasive and more objective method to diagnose acute inflammatory events prior to clinical manifestations. Because of that, it has been used in PD diagnosis (37, 96, 104, 117).

2.4 Periodontal disease in the context of orthodontic treatment

Many studies have been conducted to understand the effects of orthodontic therapy on periodontal tissues. Naranjo and collaborators (26) have evaluated modifications in the subgingival biofilm and clinical parameters after MBA insertion. Samples of the subgingival microbiota were collected from the GCF of 30 patients before and 3 months after MBA insertion. Another 30 volunteers without a fixed appliance formed the control group. The clinical status was evaluated through CAL, BOP, pocket probing depth (PPD), GI and PI. Among others, the following PP: *Aa*, *Pg*, *Pi*, *Pn*, *Tf*, *Cr*, *En*, *Fs*, *Ec* were quantified. The results showed that CAL and PPD remained constant in both groups. BOP, PI, GI increased after 3 months of MBA insertion as well as the quantity of *Pg*, *Fs* and *Tf*. For this reason, the authors concluded that the MBA insertion influences the accumulation and composition of the subgingival biofilm, leading to inflammation and bleeding on probing.

To verify the effects of MBA treatment in subgingival microflora and periodontal status among 32 adolescents (12 to 18 years old), Ristic et al. (31) assessed the concentration of *Aa*, *Pi*, *Pg* and *Fs* as well as GI, PI, PPD and CAL before and during treatment with MBA up to 6 months. Both values, clinical and microbiological parameters, began to increase after MBA insertion. The maximum values were reached 3 months after appliance insertion, followed by a decrease after 6 months. As a result, the authors concluded that MBA treatment can temporarily increases all periodontal indices and stimulate the growth of PP but without destructive effects on deep periodontal tissues.

Another study from Ristic et al. (32) also found similar results. The number of patients positive for *Pi*, *Pg* and *Fs* was significantly increased after 3 months of treatment begin but decreased after 6 months. Interestingly, *Aa* was found isolated in only one individual. Changes in PPD values were like microbiological patterns. Changes in the CAL were not significant. The results of this study confirm that MBA treatment may increase PP growth, but this increase does not lead to destructive effects on periodontal tissues.

Thornberg and co-workers (33) investigated the level of 8 bacteria (*Aa*, *Pg*, *Pi*, *Tf*, *Ec*, *Fn*, *Td*, *Cr*) associated with PD in adolescents before, during and after orthodontic treatment. After 6 months of therapy, the level of 6 analyzed PP (*Pi*, *Tf*, *Ec*, *Fn* and *Cr*) increased significantly. However, the level of these bacteria returned to normal levels 12 months after the treatment begins. The changes that occurred after this period were not statistically significant. The authors concluded that the treatment with MBA induces changes in the level of PP during and after treatment, but does not increase the risk of high levels of these bacteria.

Choi et al. (118) investigated changes in subgingival microbiota in two groups: a control group with 30 patients (mean age 16.7) who did not receive orthodontic appliances and an experimental group also with 30 patients (mean age 20) who were treated with fixed appliances. The prevalence of *Aa*, *Tf*, *Cr*, *Ec*, *Pg*, *Pi*, *Td* and *Prevotella nigrescens (Pn)* was determined at two different time points: 2 weeks before MBA removal (T1) and 3 months after MBA removal (T2). Results showed that all bacteria frequency at T1 was higher when compared to the control group, and *Tf, Cr* and *Ec* showed statistically significant differences. Nevertheless, at T2 there was no statistically significant difference between the two investigated groups. They concluded that PP during orthodontic treatment decreases significantly after MBA removal. However, 7.6% of the patients continued to present positive signs for some PP even 3 months after appliance removal.

PP increase in MBA patients was also summarized by Kim et al. (119). Thirty patients (mean age 16.7) participated in this study. Changes in subgingival microflora were observed before and during the first months (up to 6 months) of MBA treatment. *Aa*, *Tf*, *Cr*, *Ec*, *Pg*, *Pi*, *Td* and *Pn* were assessed. The results showed a significant increase in *Tf*, *Cr*, *Pn* after MBA insertion. The other PP were also increased, but not significantly. In this way, they concluded that MBA insertion affects subgingival microflora during the first months of therapy.

In 2011, van Gastel et al. (120), evaluated PPD, BOP and GCF flow changes in supra and subgingival biofilm samples of patients that needed fixed orthodontic appliance. Samples were collected at different time points: before MBA placement, and 18, 20, 24,

36 weeks of MBA insertion. All three parameters demonstrated a significant increase after bracket placement. The authors concluded that the MBA has a negative effect on microflora and clinical variables. They also published another study when they observed the patients before MBA placement (T1), direct after MBA removal (T2), and 3 months post-treatment (T3) using the same parameters (PPD, BOP, and GCF flow). They observed an increase between T1 and T2 and decrease between T2 and T3. However, the values at T3 continued higher when compared to T1.

Ghijselings et al. (121) published a follow-up study by van Gastel (120). They used the same parameters, PPD, BOP, and GCF flow, but observed until 2 years after appliance removal. The results showed an increase in all 3 parameters at debonding, although the values returned to normality at the last time point.

Liu et al. (122) evaluated changes in periodontal tissues and alterations in the *Pg* levels in two groups (A and B) of patients with an MBA. The group A was examined before appliance insertion and 3 months after appliance placement while group B was examined 1, 3, and 6 months after appliance removal. GI, PI, PPD and *Pg* concentration were evaluated. After 3 months of therapy, a significant increase could be seen in GI and PI values, which decreased 6 months after appliance removal. However, the *Pg* concentration remained higher even 6 months after MBA removal compared to Baseline. Therefore, the authors concluded that MBA treatment is favorable for dental plaque accumulation and gingival inflammation, but after its removal the periodontal status can be improved.

Kim et al. (30) evaluated the alterations occurred in the level of 5 PP (*Aa*, *Fn*, *Pg*, *Pi*, *Tf*) also clinical aspects using a GI and PI before and after the removal of the fixed appliance. Saliva samples and periodontal parameters were obtained from 54 adult patients at different time points: immediately before appliance removal (T1), 1 week (T2), 5 weeks (T3) and 13 weeks (T4) after debonding. The results demonstrated a statistically significant decrease at GI and PI immediately after debonding (T2). PP salivary levels declined at T3, while the levels of *Pi* and *Tf* decreased only at T4. In spite of, *Aa* and *Fn* amounts remained unaltered even 3 months after the fixed appliance

removal. So, the authors suggest that the risks to periodontal problems cannot be completely eliminated in the initial periods following debonding.

Guo and collaborators (11) also investigated clinical and microbiological consequences of treatment with fixed appliance in adults and children. Clinical aspects such as PI, GI, CAL and PPD and subgingival levels of *Pg*, *Fn*, *Pi*, *Tf* were recorded at 3 different time points: before treatment (T0), 1 month (T1) and 3 months (T2) after therapy begin. *Pg*, *Fn*, *Pi* and *Tf* levels increased from T0 to T2, but the differences were not significant. On the other hand, the percentage of adults with positive findings for these bacteria was significantly higher than in children at T0 and T1, but not at T2. PI and PPD also increased from T0 to T2 in both groups; but, no patient presented CAL. Thus, they concluded that fixed orthodontic appliance can negatively influence periodontal and microbiological status of both adults and children in the first few months of treatment.

A similar study conducted by Guo R. et al. (123) evaluated microbiological alterations and clinical parameters (PI, GI) in the first 3 months of MBA treatment. In this study, the investigated bacteria were *Pi*, *Cr*, *Fn* and Td. No statistically significant differences were found in subgingival microflora or in the PI. GI significantly increased after 3 months of therapy. It was concluded that the subgingival microbiota may be affected due appliance presence and may cause a mild transient gingival inflammation.

A systematic review conducted by Freitas et al. (29) investigated the existence of scientific evidence to support the hypothesis that the presence of fixed orthodontic appliances influences oral microbiota. The initial research found 305 papers, 33 of which were selected according to title and abstract. After a complete reading of the selected papers, only 8 articles met the inclusion criteria. Four of them were classified as low methodological quality articles and the other 4 as moderate articles. The authors concluded that the literature provided moderate evidence that the presence of fixed appliances influences the quantity and quality of oral microbiota.

Another systematic review (124) included 13 articles that evaluated changes in the subgingival microbiology of patients submitted to treatment with a fixed orthodontic appliance. Four PP were analyzed: *Aa*, *Pg*, *Pi* and *Tf*. After fixed appliance placement,

Pg and *Aa* frequency showed no statistically significant changes, while *Tf* increased significantly over a short period of observation (3 months). *Pi* presented a tooth-specific difference, with significant increase in the incisors but not in the molars. During longer periods, more than 6 months, 2 studies demonstrated an increased subgingival level of these bacteria, with subsequent reduction to pre-treatment levels. After appliance removal, none of the 4 bacteria showed significant differences compared to the pre-treatment values. It is, therefore, assumed that subgingival levels of bacteria show a temporary increment after appliance insertion, but they tend to decrease to pre-treatment levels after a certain period. For this reason, the fixed orthodontic appliance does not seem to induce permanent but temporary periodontal damage.

2.5 Gingival Index and Plaque Index in patients with MBA

The GI proposed by Löe and Silness in 1963 (15) remains nowadays very useful to assess the gingiva status. This index has 4 degrees: grade 0 for normal gingiva without inflammation; grade 1 to mild inflammation, with a minor color and texture changes, and no bleeding on probing; grad 2 indicates moderate inflammation with moderate redness, edema, glazing and bleeding on probing; Grad 3 means a severe inflammation, with marked redness, edema, ulceration and tendency to spontaneous bleeding.

Among plaque index, several indices were created along the years to assess plaque levels on the dental surface, such as Silness and Löe (125), modified Silness and Löe (126) O´Leary (127), Quigley and Hein (QHI) (128), Turesky Index (129), Navy Index (130). They are all based on a subjective visual plaque amount evaluation on dental surface (gingival margin and tooth crown).

Al-Anezi and Harradine evaluated methods and indices used to assess the presence of plaque in patients with MBA (10). Forty studies met the inclusion criteria and were enrolled in this review. It was found that the majority of the studies conducted with MBA patients used the original index according to Silness and Löe (31, 131-134). According to this index, there are 4 scores: 0 indicates the absence of plaque; 1 means thin film adhered along the free gingival margin; 2 indicates visible plaque; 3 indicates abundant plaque accumulation. This index refers to dental plaque progression from gingival to incisal regions.

Conventional plaque indices are intended for dental plaque assessment in patients without an MBA. Nevertheless, patients with fixed orthodontic appliance show a typical pattern of plaque accumulation that is affected by the device components, showing plaque accumulation around the bracket bases, under the arch wire and in the cervical region of the teeth near the gingival margin as was demonstrated in Klukowska et al. (9) study. Clerehugh et al. (126), Thienpont et al. (135) and Costa et al. (136) utilized a Silness and Löe modification index, which was developed to evaluate teeth with

brackets, taking into account the sites predisposed to plaque retention in MBA patients. In this index, tooth surface around the brackets base is divided into four areas: mesial, distal, incisal and gingival. The dental plaque presence in each of these four areas is evaluated according to the four scores proposed by Silness and Löe. In this way, the sum of all areas can be scored between 0 and 16.

The studies conducted by Wilcoxon et al. (137), Naranjo et al. (26), Øgaard et al. (138) and Poormoradi et al. (139) used the O´Leary index. This index evaluates the buccal and lingual surfaces and each tooth is divided into mesial, medial and distal areas. Plaque presence is then recorded for each surface, plaque amount regardless, so that each dental surface can reach a maximum score of 3. Subsequently, the index is expressed as a percentage of the total area.

Some studies have been using the QHI (140-142) and Turesky´s Quigley-Hein Index modification (mQHI) (123, 143, 144) for dental plaque assessment. The QHI evaluates supragingival plaque distribution on dental surfaces after staining with a plaque revelator (Table 1). The mQHI represents its variation and determines plaque distribution on the proximal and buccal surfaces. Plaque revelators can be used to show patient's oral hygiene problematic areas, stimulating and motivating them to reach a better oral hygiene.

Table 1: Quigley and Hein Index: scores and criteria (128)

Score	Criteria
0	No Plaque
1	isolate areas plaque at gingival margin
2	Thin line of plaque at gingival margin (≤1 mm)
3	Gingival third of tooth surface is covered with plaque
4	Two thirds of tooth surface is covered with plaque
5	More than two thirds of the tooth surface is covered with plaque

In a study carried out by Kossack and Jost-Brinkmann a modification of the mQHI was used to better graduate the stained dental plaque in the presence of the MBA (145). Figure 4 illustrates the mQHI scores.

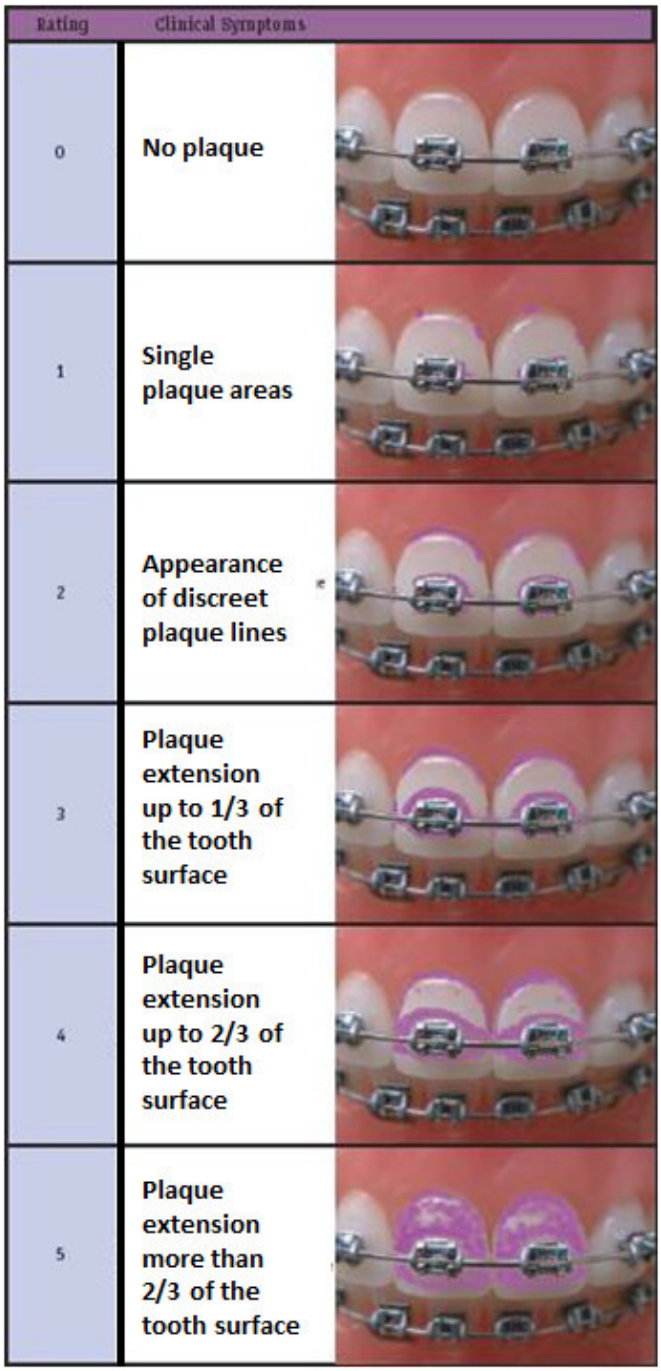


Figure 4: Scores of the modified Quigley-Hein index in patients with fixed orthodontic appliance. Photo: Article: Kossack and Jost-Brinkmann (145).

Studies conducted by Kiliçoğlu et al. (146), Burden et al. (147) and Türkkahraman et al. (148) used the Bonded-Bracket-Plaque-Index to evaluate dental plaque amount. Plaque assessment occurs in six grades and takes into account bracket presence on tooth buccal surface.

Atassi and Awartani investigated in their study oral hygiene among orthodontic patients using Ortho-Plaque Index (149). Klukoswka et al. worked with digital image plaque analyses to estimate plaque percentage coverage and confirmed elevated rates of plaque deposition behind an arch wire and around the braces (9).

3. Materials and Methods

This prospective clinical study was conducted at the Orthodontics Department of Johannes Gutenberg University of Mainz, Germany. All study participants were patients of this clinic.

All volunteers and their legal guardians received written and orally information about the exact procedure and study objectives and signed a declaration of consent (see Appendix – Figure 30) before the study began. Then they completed two anamnesis formularies, one from the University Medicine Mainz - Clinic for Dental, Oral and Maxillofacial Diseases (see Appendix – Figure 31) and another one from the Orthodontics Department of the Johannes Gutenberg University of Mainz (see Appendix – Figures 32,33).

3.1 Subjects

The subjects for the study were selected after the positive vote of the Ethics Committee (number 837.340.12 (8441-F)). From a clinic currently patients list, 80 patients were blindly selected to receive an orthodontic fixed appliance from buccal in the upper jaw (UPJ) and lower jaw (LOJ). They were asked by the study examiner if they wished to participate in this study either by telephone or directly at an examination appointment in the clinic. 55 subjects were included in this study (30 females, 25 males) aged 12 to 17 years. The average age of the patients was 13.81 ± 1.3 years. Participation was independent of gender or ethnicity.

3.1.1 Inclusion Criteria

All study subjects recruited fulfilled the following inclusion criteria: had a malocclusion, which had to be corrected with an orthodontic fixed appliance (conventional metallic brackets) in the UPJ and LOJ; a minimum of 16 natural teeth, including 8 anterior teeth; good general and periodontal health.

The last teeth brushing must have taken place before 8:00 a.m. on the study day. Eating, drinking or smoking was allowed up to two hours before the examination visit. Up to 45 minutes before the appointment, only a sip of water was allowed.

The participants were instructed to maintain their previous manual or electrical cleaning habits. They had to regular attend the study´s appointment. For the duration of this study, the volunteers were not allowed to participate in any other clinical study.

3.1.2 Exclusion Criteria

Subjects were excluded of this study if they had a previous therapy with an orthodontic fixed appliance in UPJ and LOJ or subjects who did not require orthodontic treatment; more than 3 carious defects; a severe periodontal disease characterized by purulent exudate, tooth mobility and/or gingival recession likewise, subjects undergoing periodontal treatment.

They could not participate if they had a treatment with ceramic brackets, lingual brackets or removable appliance; specifics allergies to dyes/colorants used in cosmetic products, food or allergies to colorant used as dental diagnostic procedures (Mira-2 –Ton; Hager & Werken). Also, patients who had taken antibiotics two weeks before study start or a professional dental cleaning were excluded.

Patients with a chronic medical disease, pacing, syndromes and general diseases, as well as pregnant patients were also not allowed to participate in the study.

3.1.3 Continuance Criteria

The participants were repeatedly instructed to brush their teeth before 8:00 a.m. and to eat, drink or smoke for the last time at least two hours before the appointment. They could drink water until 45 minutes before the appointment. In addition, they were not allowed to participate in any other study. In the course of the study, the volunteers were forbidden to take antibiotics two weeks before the appointment. Professional dental cleaning could not be performed for the entire period.

3.2 Overall Study Design

The entire study consisted of six time points. The examinations occurred between 8 a.m. and 5 p.m., the regular opening hours of the clinic. Each time point was scheduled for half an hour. The schedule was as follows:

- Baseline (T0): 1 week before bracket bonding
- Time 1 (T1): 3 weeks after bracket bonding
- Time 2 (T2): 6 weeks after bracket bonding
- Time 3 (T3): 3 months after bracket bonding
- Time 4 (T4): 6 months after bracket bonding
- Time 5 (T5): 1 year after bracket bonding

Baseline (T0)

The baseline (T0) was performed one week before bracket bonding. First, an oral and perioral cavities visual examination was conducted using a dental mirror and a standard dental light. Teeth, gingival (free and attached), hard and soft palate, labial mucosa, oropharynx/uvula, tongue, mouth floor, and lips were examined. All abnormal findings were noted and categorized by their location. Afterwards the gingival index (GI) according to Löe and Silness (15) and Turesky modified plaque index (PI) according to Quigley-Hein (145) were conducted. Then, the patients brush their teeth with a manual

toothbrush (Oral-B® indicator 35 soft; Procter & Gamble, Kronberg, Germany) and toothpaste (Blend-a-med Classic 1450 ppm sodium fluoride; Kronberg, Germany). Subsequently, the GCF samples were collected. Adverse events and general comments were noted.

The insertion of the fixed orthodontic appliance (bracket bonding) was one week after baseline (T0) by the treating dentist. All teeth were cleaned with polishing paste without fluoride (Zircate Prophy Paste, Dentsply/Maillefer, Ballaigues, Switzerland). The teeth were then prepared for bonding using the acid etching technique (Unitek Etching Gel 3M, California, USA) and Transbond XT Light Cure Adhesive Primer (Unitek 3M, Monrovia, USA). Subsequently, the metallic brackets, nickel-free, system-slot 0.022" (Micro Sprint Brackets - Forestadent®; Pforzheim, Germany) were bonded with Transbond XT Light Cure Adhesive in capsules (Unitek 3M, Monrovia, USA) both in the UPJ and LOJ. On the first molars, bands were cemented (Ketac Glass Ionomer Luting Cement 3M ESPE, Neuss, Germany). On this day, the volunteers received oral hygiene and cleaning instructions (Figure 34, see Appendix) from the Department of Orthodontics at Mainz University Medical School and a prescription of Elmex Gelée (Colgate-Palmolive®; Hamburg, Germany), which they had to use once a week.

T1 occurred 3 weeks after fixed orthodontic appliance insertion of the The first step was to check the continuance criteria by the participants. A dental examination was performed to examine the oral cavity and to record the GI and PI values. Then, the participants had to brush their teeth. Finally, GCF samples were taken. Adverse events and general comments were noted.

T2 occurred 6 weeks after fixed orthodontic appliance insertion. The procedure at T2 was the same procedure as at T1.

T3 took place 3 months after fixed orthodontic appliance insertion. The procedure at T3 was the same as at T1 and T2.

T4 happened 6 months after fixed orthodontic appliance insertion. Once again, a dental examination was performed to examine the oral cavity. Afterward, GI and PI values were collected. GCF samples were taken. In addition to the other time points, the patient received professional teeth cleaning and a new prescription for Elmex Gelée. The participants received once again oral hygiene and cleaning instructions (Figure 34, see Appendix).

The last time point (T5) occurred 1 year after the fixed orthodontic appliance insertion and was scheduled according to T4. Table 2 shows a summary of the study schedule.

Table 2: Study schedule by procedures according to different time points

Study Plan	T0	T1	T2	T3	T4	T5
Informed Consent	X					
Medical History	X					
Inclusion / Exclusion Criteria	X					
Continuance Criteria		X	X	X	X	X
Oral Tissue Examination	X	X	X	X	X	X
Gingival Index	X	X	X	X	X	X
Plaque Index	X	X	X	X	X	X
GCF-Sample	X	X	X	X	X	X
Professional teeth cleaning					X	X
Oral Hygiene Instructions / Aid Distribution					X	X
Rezept Elmex Gelée					X	X
General Comments	X	X	X	X	X	X
Adverse Events		X	X	X	X	X

TO → 1 week before bracket bonding
T1 → 3 weeks after bracket bonding
T2 → 6 weeks after bracket bonding
T3 → 3 months after bracket bonding
T4 → 6 months after bracket bonding
T5 → 1 year after bracket bonding

3.3 Clinical Indices

3.3.1 Gingival Index

The gingival color assessment, consistency, inflammation and bleeding on probing was performed using the Löe and Silness index (15). All teeth except the molars were assessed. Using a mouth mirror, a periodontal probe and an appropriate light all teeth were examined and scored. Each tooth was divided into six gingival areas: distobuccal, buccal, mesiobuccal, mesiolingual (or mesiopalatinal), lingual (or palatinal) and distolingual (or distopalatinal). Teeth and gingiva were gently dried with air before scoring to provide proper visibility. Then, without pressure, the periodontal probe tip was inserted about 1 mm into the gingival margin. Each of the six tooth surfaces received a score of 0-3. Unscorable tooth received the number 8 and missing tooth number 9. An index for entire mouth is determined by dividing the total score by the number of surfaces examined. The evaluation grades of the GI are listed in Table 3.

Table 3: Gingival Index: scores and criteria

Score	Criteria
0	Normal Gingiva.
1	Mild inflammation – slight change in color, slight edema; no bleeding on probing.
2	Moderate inflammation – redness, edema and glazing; bleeding on probing.
3	Severe inflammation – marked redness, edema and Ulceration; tendency to spontaneous bleeding.
8	Unscorable tooth.
9	Missing tooth.

3.3.2 Plaque Index

All teeth were stained with a foam pellet (Erkodent®/Pfalzgrafenweiler, Germany) and a plaque disclosing agent (Mira-2-Ton, Hager Werken/Duisburg, Germany), which makes plaque visible. Subjects then rinsed the mouth thoroughly with water and supragingival plaque was evaluated at 6 sites (distobuccal, midbuccal, mesiobuccal, distolingual (or distopalatinal), midlingual (or midpalatinal) and mesiolingual (or mesiopalatinal) (Figure 5). Molars, crowns and surfaces with cervical restorations were not evaluated. For each subject, a whole mouth average plaque score was assessed. To calculate total plaque level, the total score was divided by the number of examined teeth. The modification of mQHI proposed by Kossack and Jost-Brinkmann (145) was utilized to assess the plaque level on the buccal and lingual/palatinal surfaces of all teeth.

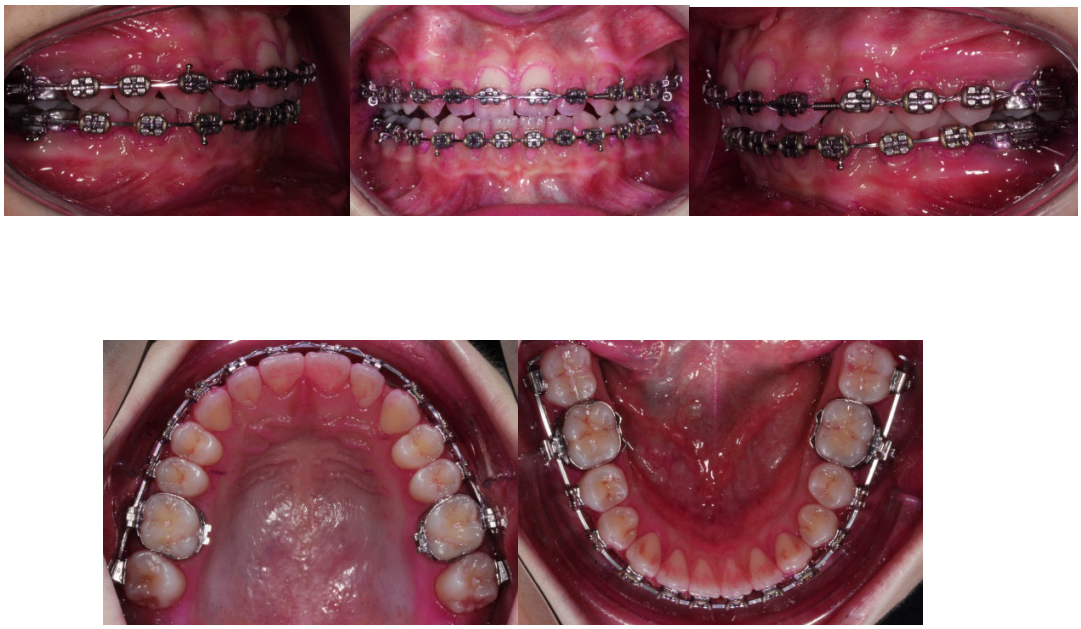


Figure 5: Patient with orthodontic fixed appliance; teeth stained with a plaque disclosing agent (Plaquerevalator: Mira-2-Ton®, Hager & Werken, Duisburg, Germany); Photos: P. Ferrari Peron.

3.4 Subgingival Samples

3.4.1 Subgingival Samples – Periodontopathogens

The teeth 16, 12, 24, 36, 32, 44 were selected to represent the entire set of teeth for sampling the GCF. These teeth were chosen based on the Ramfjord system, in which the group of teeth (incisors, premolars and molars) has its representative (150-152), as illustrates in Figure 6. If a Ramfjord tooth was missing, a substitute tooth (teeth number: 17, 11, 25, 37, 31, 45) was chosen as proposed by Fleiss et al.(153).

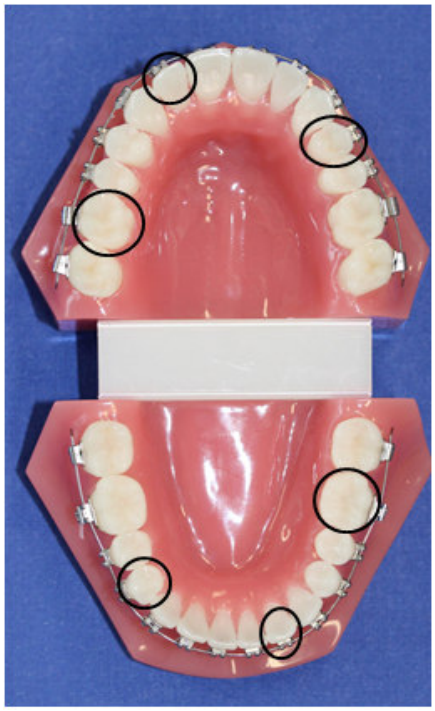


Figure 6: Ramfjord teeth; photo/edited: P. Ferrari Peron.

The area was gently dried and with cotton rolls (Roeko-Luna; Coltène-Whaledent Gmbh, Langenau, Germany) isolated from oral fluid to avoid paper tips contamination. With a clamp and sterilized absorbent paper points (Number 40 – Absorbent Paper Points, Dentsply International®/ York, USA) the GCF was collected at the mesial sides of the selected teeth for 30 seconds. The paper points were dropped and stored in a sterile and dry tube (1.5ml natural flat cap DNAs and RNAs free micro centrifuge tubes,

Eppendorf Tube, Ahrensburg, Germany) (Figure 7), packed in a plastic box and in an envelope provided by Bioscientia. On the same day, the probes were sent using DHL Express service to the Bioscientia Laboratory.

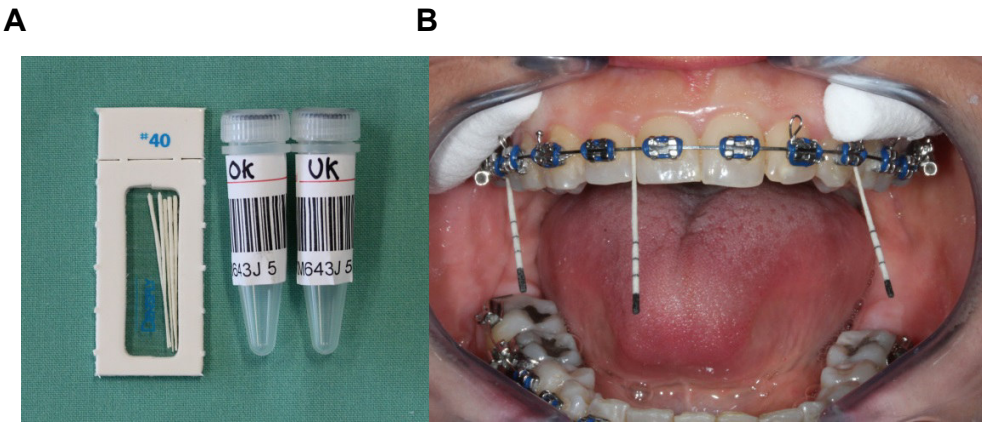


Figure 7: (A): Paper points and tubes; (B): Patient during test admission; Photo: P. Ferrari Peron.

The analysis was carried out using a polymerase chain reaction (PCR) and detection methods with gene probes. The following 11 PP were examined: *Aa*, *Pg*, *Tf*, *Td*, *Fs*, *Pm*, *Pi*, *Cr*, *En*, *Ec*, *Cs* (Figure 8).

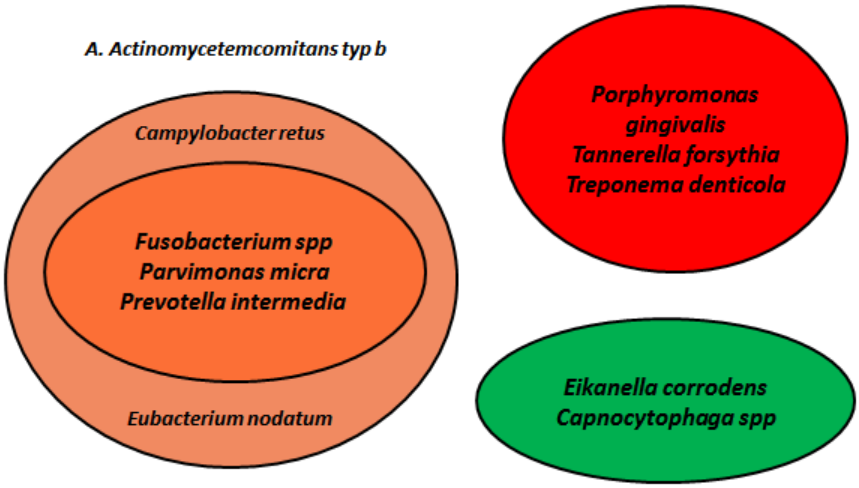


Figure 8: The 11 periodontopathogens investigated in this study (microbial subgingival complex according to Socransky et al. (72)); Edited: P. Ferrari Peron.

3.4.2 Subgingival Samples – Concentration of aMMP-8

Using a sterilized paper strip (GCF collection Strips – dentognostics GmbH; Jena, Germany), GCF was removed as the same way as from PP but from the distal approximal space of the above mentioned teeth for aMMP-8 examination (Figure 9). These were also collected as pool samples from the UPJ and the LOJ. Afterward, the paper strips were stored in a sterile and dry tube (1.5ml natural flat cap DNAs and RNAs free microcentrifuge tubes, Eppendorf Tube, Ahrensburg, Germany) and sent to Bioscientia Laboratory (Figure 10). The samples were quantitatively analyzed for aMMP-8 using an enzyme-linked immunosorbent assay (ELISA) in Bioscientia Laboratory. Table 4 lists the evaluation grades according to Bioscientia labor.

A **B**

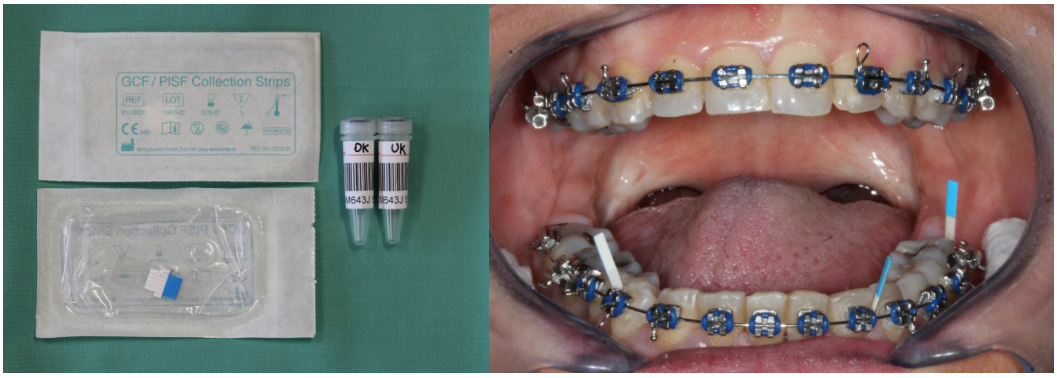


Figure 9: (A): GCF paper strip and tubes; (B): Patient during test admission; Photo: P. Ferrari Peron.

Figure 10: (A): Sampling set for the detection of periodontopathogens and the concentration of aMMP-8; (B): Envelope from the company Bioscientia. Photo: P. Ferrari Peron.

Table 4: Degree of evaluation of aMMP-8 concentration

Level	Criteria
<1<8ng	healthy/not inflamed
8<20ng	Low degree of inflammation/no increased risk of progressive periodontal tissue loss
20ng	acute inflammation/ strong risk for periodontal tissue loss

3.5 Laboratory analyses

All the laboratory analyses were performed in Bioscientia laboratory (Institut für Medizinische Diagnostic GmbH, Berlin). The informationen regarding the procedures and instructions used for the analyses were provided by the laboratory. According to the two types of samples mentioned above, the following tests were performed: 11 PP analysis and aMMP-8 determination.

3.5.1 Analysis of the periodontopathogens

To evaluate the presence of PP in GCF samples, bacterial DNA nucleic acids were manually extracted from the GCF samples obtained with paper using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany). The 11 PP were analyzed in parallel and qualitatively with the DNA-based in vitro detection system LCD Array Kit BAC-Dent 2.4 (version: BAC-Dent 2.4 CE V-7.0-2013-GER, Chipron GmbH, Berlin, Germany). To amplify bacterial 16S rRNA gene small regions from the extracted bacterial DNA, polymerase chain reaction (PCR) was performed. The PCR master mix contained three primer mixtures (triplex PCR) to act directly against three different bacterial 16S rRNA gene regions (Primer Mix PA300 - 350 Bp, Primer Mix PA500 - 85 Bp, Primer Mix PA1000 - 300 Bp). The resulting DNA fragments were labeled with biotin during PCR.
Subsequently, the labeled PCR amplicons surface were hybridized to species specific capture probes immobilized on LCD-Chip. In this way, all 11 PP could be simultaneously differentiated and detected in a single reaction. The PCR amplicons were mixed with a hybridization buffer (per reaction 22 µl hybridization buffer B, 2 µl modulator and 10 µl PCR amplificate) and 28 µl of this solution was pipetted into an array field. The chip was incubated at 35 °C in the water bath for 30 min. Then, the PCR amplicons binded to specific probes at the array chamber bottom and unspecific amplicons were removed by washing steps (3 wash containers with 150 ml single wash buffer working solution, 10 s rinsed in wash containers 1 and 2 and incubated for 1 min in wash container 3; 15 s chip dried by centrifugation). The visualization of bound amplicons is mediated by an enzyme-substrate cascade. The specifically bound biotinylated PCR fragments incubated with streptavidin peroxidase conjugate remained. For this purpose, 28 µl of a

label mixture (27 µl Dilution Buffer, 3 µl Modulator, 0.2 µl Label) were pipetted into the array fields and incubated at room temperature for 5 min. After a further wash (as before), 28 µl staining solution was pipetted to each array field and allowed to react for 5 min. The staining was stopped by immersing the chip for 10 s in the third wash solution from the previous wash. The specific DNA fragments were visible by substrate precipitation (dark blue precipitation of the converted substrate stain). The analysis was performed automatically on a PC using a Slide Scanner and the Slide Reader software (Analysis Package, Chipron GmbH, Berlin, Germany). Due to the separate pool samples, the results of LOJ and UPJ were also presented separately. The laboratory results were sent by post in the form of standardized findings sheets and were present as follow:

0: no PP is verified – (<104 KbE)
1: Low PP concentration (+) (=104 KbE)
2: Increased PP concentration + (<105 KbE)
3: Strongly increased PP concentration ++ (<106 KbE)
4: Extremely increased PP concentration +++ (>107 KbE)
The definition of the microorganism concentration was the same for all pathogens, except for *A. actinomycetemcomitans* that the values with a power of ten were considered lower (e.g.: (+) = 104 KbE).

3.5.2 Determination of active MMP-8

Total aMMP-8 was quantitatively determined in the GCF samples obtained with paper strips using ELISA (dentoELISA aMMP-8, dentognostics GmbH, Jena, Germany). The GCF eluate was obtained from the strip sample using 600 µl phosphate buffer per strip. The tubes were vortexed, left at room temperature for 5 min, and vortexed again before removing the strips. Then, eluate was diluted in a ratio of 1:50 (10 µl GCF eluate + 490 µl phosphate buffer). Subsequently, 100 µl of each: calibrators 1-5 (aMMP-8 concentrations of 0.125 ng/ml, 0.25 ng/ml, 0.5 ng/ml, 1.0 ng/ml, 1.6 ng/ml), dilution buffer (phosphate buffer), positive control (0.75 ng/ml aMMP-8) and the sample eluate in duplicate were pipetted into the corresponding wells of the microtitration plates. The plate was closed and incubated at 37 °C for 60 min. Specific antibodies against the active matrix metalloproteinase-8 were fixed at the bottom of the wells of this plate. The aMMP-8 from the GCF also from the calibrators formed immune complexes with those fixed antibodies. The unbound components were removed by washing three times with 200 µl per well of wash buffer each time. Subsequently, 100 µl of peroxidase enzyme conjugate (monoclonal anti-aMMP-8 antibody coupled with peroxidase) was added to each well and incubated at 37 °C for 30 min. The same washing process was performed to remove unbound components. The conjugate combined with the fixed immune complexes remained at the well bottom and was stained using 100 µl of TMB substrate (TMB = 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide) and incubated on a shaker (500 rpm) for 15 min. Peroxidase produces a blue dye and after the addition of a stop solution (0.25 mol/l sulfuric acid; 100 µl per well) the reaction turns yellow. Color intensity is proportional to the concentration of aMMP-8 in the sample. The plate was evaluated in a microtitration plate photometer at 450 nm (reference wavelength 620 nm). aMMP-8 analysis results were given in nanograms per milliliter (ng/ml). Lower and upper limits of 8 ng/ml and 20 ng/ml, respectively, were defined. All values below 8 ng/ml were defined as "low range, healthy, not inflamed". Values ≥ 8 ng/ml were defined as "inflammatory events in the sampling area". The range between 8 ng/ml and 20 ng/ml was described as "mildly inflamed" and values ≥ 20 ng/ml as "acute inflammation".

3.6 Statistical Analysis

The following programs were used for statistical analysis:
- SPSS (IBM® SPSS® Statistics, Version 23, IBM Corporation, Armonk, USA, © 1989, 2015)
- Software SAS 9.4 (SAS Institute Inc., Cary, North Carolina, USA, © 2002-2012)
- Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA)

The mean value (MV) and the standard deviation (SD) were determined for the assessment and evaluation of the GI, PI and aMMP-8 values at the respective time points. In addition, the data were presented with details of the interquartile distance (IQD) and the median in tabular form and as box plot diagrams. In summary, statistics (MV, SD, IQD, median) were calculated for each time point.

The statistical evaluation of the concentration of the periodontopathogens was performed with the computer software SAS. The Excel program was used to graphically illustrate the results in form of bar charts.

Changes of GI, PI, and aMMP-8 over time and differences between upper and lower jaw were assessed using paired t-tests comparing values at follow-up visits to baseline values. Trends in the prevalence and concentration of bacteria were assessed using the sign test.

The significance level was chosen as α=0.05. As numerous comparisons were performed and focus was on detecting possible changes and associations no formal adjustment for multiple testing was performed. Therefore, only the local significance level was controlled and the probability of obtaining at least one false positive result is substantially higher than 5%.

4. Results

4.1 Subjects

A total of 55 individuals were screened for this study and 50 participants completed the study. One subject had the MBA removed before 1 year was completed and therefore could not have the 6th appointment. One individual moved to another city, and changed dentist and could not participate in the study anymore. Another subject went to an exchange program in the USA and missed the last appointment. Two other subjects showed a lack of compliance and both were excluded after the 5th time point.

Fifty-four subjects were Caucasians (98.2%) and one subject was of Asian-oriental origin (1.8%). Twenty five subjects (45.5%) were male and thirty were female (54.5%). The demographic characteristics are shown in Table 5.

Table 5: Demographic characteristics of the subjects

Age (years)	**N=55**	
Mean value	13,8	
SD	1,29	
Minimum	12	
Maximum	17	
Ethnicity	**Frequency**	**Percentage**
ASI (Asian-oriental)	1	1,8%
CAUC (caucasia)	54	98,2%
Total (n)	55	100%
Gender	**Frequency**	**Percentage**
Male	25	45,5%
Female	30	54,5%
Total (n)	55	100%

SD: Standard Deviation

4.2 Gingival Index

The results are presented first as the total gingival index in UPJ and LOJ at different time points. After that, GI was presented separately in the UPJ and LOJ per visit and a comparison between GI – UPJ and GI – LOJ.

4.2.1 Total Gingival Index

The total GI at baseline (T0) for UPJ and LOJ scored 0.34, which means normal gingiva. A slight decrease in GI was observed at T1 followed by an increase at T2 ($p<0.005$). Afterwards GI gradually significantly increased ($p<0.0001$) until T5 when it had reached its maximum peak and scored 0.95 signaling a tendency to gingival inflammation. The total GI values for all time points are shown in Figure 11 and Table 6.

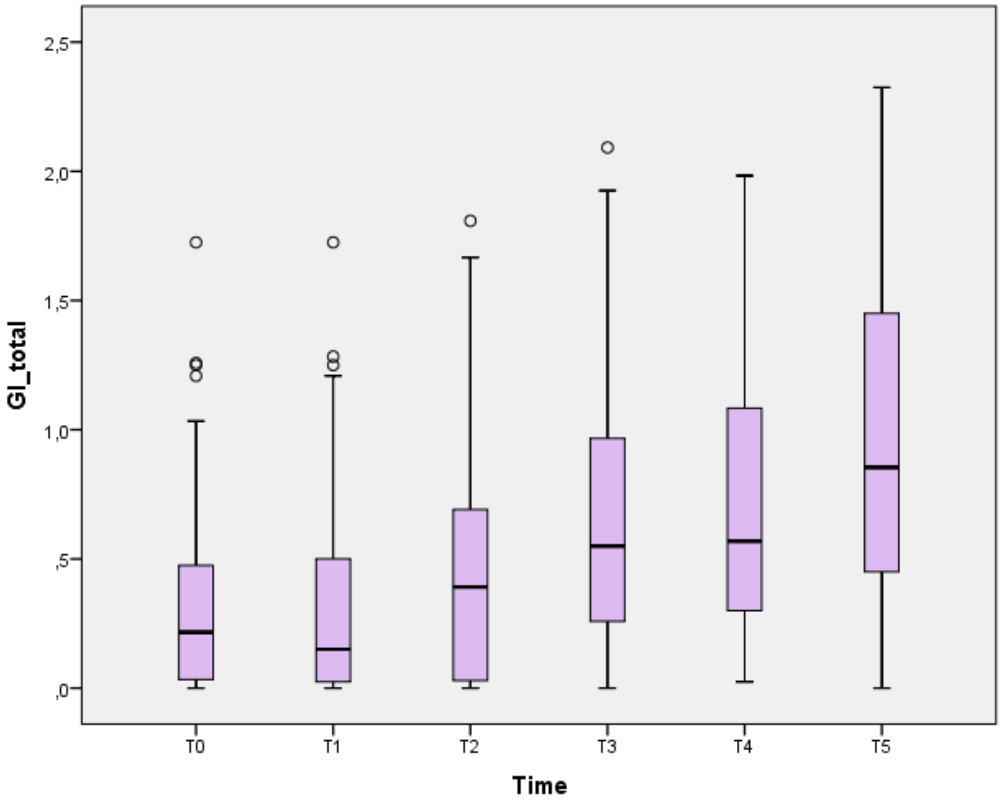


Figure 11: Boxplot diagram showing the gingival index for the entire upper and lower jaws at different time points.

Table 6: Statistical parameters of the total gingival index at different time points

GI – Total	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean changes from baseline (SD)	p-value
Baseline (T0) **(N=55)**	0.03	0.34 (0.39)	0.22	0.48	0 – 1.73	NA	NA
Time 1 (T1) **(N=53)**	0.03	0.32 (0.40)	0.15	0.50	0 – 1.73	-0.01 (0.19)	0.6718
Time 2 (T2) **(N=55)**	0.03	0.48 (0.48)	0.39	0.73	0 – 1.81	0.14 (0.36)	0.0063*
Time 3 (T3) **(N=55)**	0.25	0.68 (0.53)	0.55	1.00	0 – 2.09	0.34 (0.45)	0.0001**
Time 4 (T4) **(N=54)**	0.30	0.72 (0.54)	0.57	1.08	0 – 1.98	0.38 (0.49)	0.0001**
Time 5 (T5) **(N=50)**	0.45	0.95 (0.60)	0.85	1.45	0 – 2.33	0.62 (0.62)	0.0001**

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA; * $p<0.05$; ** $p<0.0001$

4.2.2 Gingival Index – comparison between upper and lower jaw

In UPJ there was a small variation in the GI value from T0 until T1, with a decrease at T1. A statistically significant increase of the GI value occurred at T2 ($p<0.05$) and then a continuous increase was observed until T5 ($p<0.0001$). Table 7 illustrates the GI values at different time points in UPJ.

The same variation was noted in LOJ. There was a statistically significant increase of the GI value at T2 ($p<0.05$) with continuous increase until T5 ($p<0.0001$). Table 8 shows the GI values for LOJ.

There was no statistically significant difference between UPJ and LOJ. Figure 12 illustrates the total GI for UPJ and LOJ at different time points and table 9 shows a comparison between UPJ and LOJ.

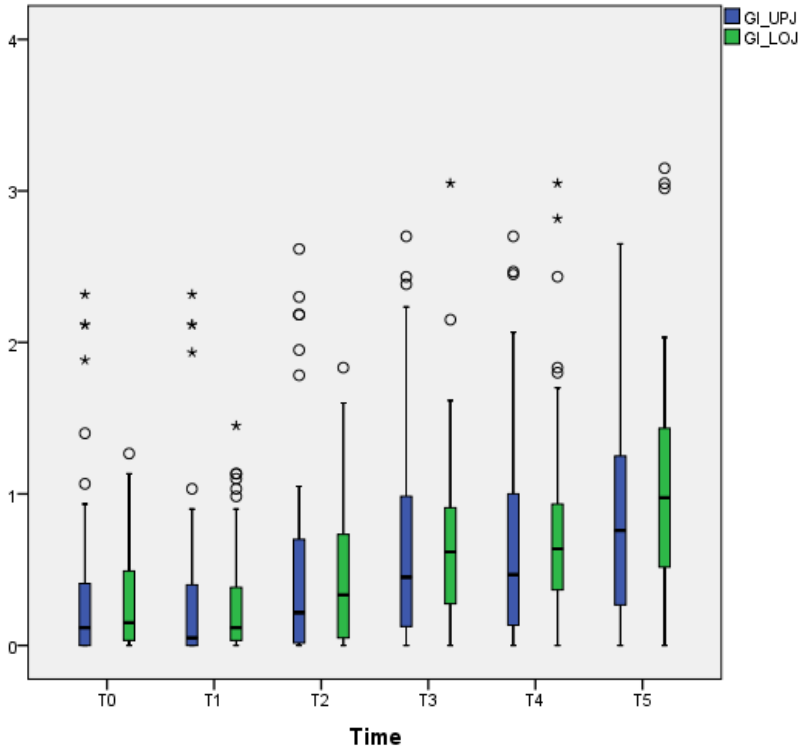


Figure 12: Boxplot diagram illustrating the gingival index for upper and lower jaw separated at different time points.

Table 7: Statistical parameters of gingival index for upper jaw at different time points

GI – UPJ	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean changes from baseline (SD)	p-value
Baseline (T0) **(N=55)**	0.00	0.36 (0.59)	0.12	0.43	0 – 2.32	NA	NA
Time 1 (T1) **(N=53)**	0.00	0.35 (0.58)	0.12	0.40	0 – 2.32	-0.01 (0.27)	0.7136
Time 2 (T2) **(N=55)**	0.02	0.50 (0.67)	0.22	0.72	0 – 2.62	0.14 (0.42)	0.0152*
Time 3 (T3) **(N=55)**	0.10	0.67 (0.72)	0.45	1.00	0 – 2.70	0.31 (0.53)	<.0001**
Time 4 (T4) **(N=54)**	0.13	0.68 (0.70)	0.47	1.00	0 – 2.70	0.31 (0.50)	<.0001**
Time 5 (T5) **(N=50)**	0.27	0.86 (0.68)	0.76	1.25	0 – 2.65	0.51 (0.60)	<.0001**

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001.

Table 8: Statistical parameters of gingival index for lower jaw at different time points

GI – LOJ	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean changes from baseline (SD)	p-value
Baseline (T0) **(N=55)**	0.03	0.32 (0.36)	0.15	0.50	0 – 1.27	NA	NA
Time 1 (T1) **(N=53)**	0.03	0.30 (0.38)	0.12	0.38	0 – 1.45	-0.01 (0.21)	0.7741
Time 2 (T2) **(N=55)**	0.05	0.45 (0.47)	0.33	0.75	0 – 1.83	0.13 (0.44)	0.0282*
Time 3 (T3) **(N=55)**	0.27	0.69 (0.55)	0.62	0.92	0 – 3.05	0.38 (0.54)	<.0001**
Time 4 (T4) **(N=54)**	0.37	0.77 (0.65)	0.64	0.93	0 – 3.05	0.45 (0.69)	<.0001**
Time 5 (T5) **(N=50)**	0.52	1.05 (0.75)	0.98	1.43	0 – 3.15	0.73 (0.81)	<.0001**

SD: Standard deviation; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001.

Table 9: GI – Comparison between upper and lower jaw

GI	UPJ MV (SD)	LOJ MV (SD)	UPJ *vs* LOJ MV (SD)	p-value
Baseline (T0) **(N=55)**	0.36 (0.59)	0.32 (0.36)	0.04 (0.58)	0.5895
Time 1 (T1) **(N=53)**	0.35 (0.58)	0.30 (0.38)	0.05 (0.58)	0.4949
Time 2 (T2) **(N=55)**	0.50 (0.67)	0.45 (0.47)	0.05 (0.65)	0.5642
Time 3 (T3) **(N=55)**	0.67 (0.72)	0.69 (0.55)	-0.02 (0.69)	0.8216
Time 4 (T4) **(N=54)**	0.68 (0.70)	0.77 (0.65)	-0.10 (0.82)	0.3930
Time 5 (T5) **(N=50)**	0.86 (0.68)	1.05 (0.75)	-0.19 (0.78)	0.0869

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

4.3 Plaque Index

The results are presented first as the total plaque index in UPJ and LOJ at different time points. Subsequently, PI was presented separately in the UPJ and LOJ per visit as well a comparison between PI – UPJ and PI – LOJ.

4.3.1 Total Plaque Index

Before the MBA treatment begin PI showed almost the same values for all subjects in UPJ and LOJ. Total PI at T0 was with score 2.00 assessed, which means a thin continuous plaque band up to 1 mm of plaque at the cervical gingival margin. After appliance installation, the PI values continued to increase until it reached its maximum peak at T3 ($p<0.05$), 3 months later. Then, there was a small decrease at T4 (6 months after MBA) followed by an increase at T5 (1 year after MBA) ($p<0.05$). Figure 13 shows the total GI variations at different time points and table 10 summarizes the PI values.

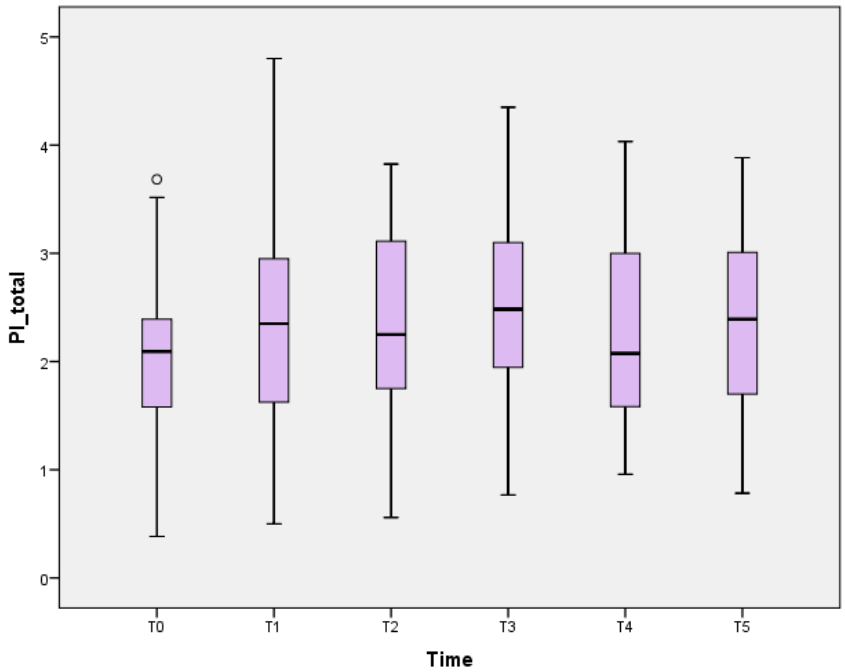


Figure 13: Boxplot diagram showing the plaque index for the UPJ and LOJ at different time points.

Table 10: Plaque index total

PI – Total	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean changes from baseline (SD)	p-value
Baseline (T0) **(N=55)**	1.57	2.03 (0.68)	2.09	2.40	0 – 4	NA	NA
Time 1 (T1) **(N=53)**	1.57	2.29 (0.92)	2.35	2.96	0 – 5	0.27 (0.86)	0.0279*
Time 2 (T2) **(N=55)**	1.75	2.38 (0.87)	2.25	3.12	1 – 4	0.35 (0.89)	0.0055*
Time 3 (T3) **(N=55)**	1.93	2.48 (0.82)	2.48	3.11	1 – 4	0.44 (0.80)	0.0002*
Time 4 (T4) **(N=54)**	1.58	2.23 (0.80)	2.08	3.00	1 – 4	0.20 (0.75)	0.0533
Time 5 (T5) **(N=50)**	1.69	2.39 (0.83)	2.39	3.03	1 – 4	0.35 (0.96)	0.0126*

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * $p<0.05$; ** $p<0.0001$

4.3.2 Plaque Index – comparison between upper and lower jaw

Table 11 shows the PI values in UPJ before and during treatment with MBA. The PI at T0 was 1.90. Thereafter, PI values continually increased until T3 ($p<0.05$), reaching its maximum peak, followed by a decrease at T4. At T5 PI increased again but without significantly difference.

PI in LOJ was 2.13 at baseline. The average PI value showed an increase after the insertion of the MBA at T2 ($p<0.05$), with a maximum PI at T3 ($p<0.0001$), followed by a decreased at T4. At the end of the observed period (T5), the PI value increased again but without significantly difference (Table 12). Comparison between UPJ vs. LOJ shows that at T1 and T2 the PI values in UPJ were higher than in LOJ. Otherwise all other PI values were higher in LOJ (Table 13). There was no significantly difference between UPJ and LOJ. Figure 14 shows the GI variations in UPJ and LOJ at different time points.

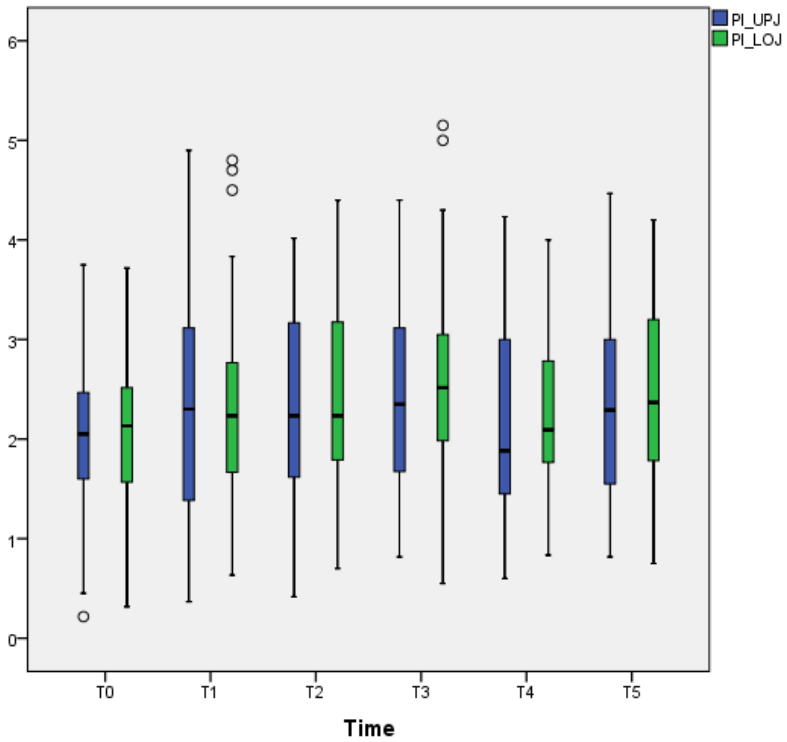


Figure 14: Boxplot diagram to illustrate the plaque index for maxilla and mandible separated at different time points.

Table 11: Statistical parameters of plaque index values in UPJ at different time points

PI – UPJ	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean changes from baseline (SD)	p-value
Baseline (T0) **(N=55)**	1.58	2.02 (0.76)	2.05	2.48	0 – 4	NA	NA
Time 1 (T1) **(N=53)**	1.36	2.23 (0.99)	2.30	3.13	0 – 5	0.23 (0.90)	0.0714
Time 2 (T2) **(N=55)**	1.60	2.33 (0.94)	2.23	3.25	0 – 4	0.31 (0.93)	0.0161*
Time 3 (T3) **(N=55)**	1.67	2.38 (0.92)	2.35	3.17	1 – 4	0.36 (0.92)	0.0049*
Time 4 (T4) **(N=54)**	1.44	2.19 (0.97)	1.88	3.00	1 – 4	0.19 (0.84)	0.1075
Time 5 (T5) **(N=50)**	1.55	2.29 (0.91)	2.29	3.00	1 – 4	0.27 (0.98)	0.0624

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001

Table 12: Statistical parameters of plaque index values in UPJ at different time points

PI – LOJ	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean changes from baseline (SD)	p-value
Baseline (T0) **(N=55)**	1.53	2.05 (0.70)	2.13	2.55	0 – 4	NA	NA
Time 1 (T1) **(N=53)**	1.66	2.34 (0.94)	2.23	2.80	1 – 5	0.31 (0.91)	0.0169*
Time 2 (T2) **(N=55)**	1.75	2.43 (0.94)	2.23	3.35	1 – 4	0.38 (1.01)	0.0071
Time 3 (T3) **(N=55)**	1.98	2.57 (0.91)	2.52	3.10	1 – 5	0.52 (0.88)	<.0001**
Time 4 (T4) **(N=54)**	1.76	2.28 (0.79)	2.09	2.78	1– 4	0.22 (0.83)	0.0606
Time 5 (T5) **(N=50)**	1.78	2.47 (0.88)	2.37	3.20	1 – 4	0.42 (1.11)	0.0096

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001

Table 13: PI – Comparison between UPJ and LOJ

PI	UPJ MV (SD)	LOJ MV (SD)	UPJ *vs* LOJ MV (SD)	p-value
Baseline (T0) **(N=55)**	0.36 (0.59)	0.32 (0.36)	0.04 (0.58)	0.5895
Time 1 (T1) **(N=53)**	0.35 (0.58)	0.30 (0.38)	0.05 (0.58)	0.4949
Time 2 (T2) **(N=55)**	0.50 (0.67)	0.45 (0.47)	0.05 (0.65)	0.5642
Time 3 (T3) **(N=55)**	0.67 (0.72)	0.69 (0.55)	-0.02 (0.69)	0.8216
Time 4 (T4) **(N=54)**	0.68 (0.70)	0.77 (0.65)	-0.10 (0.82)	0.3930
Time 5 (T5) **(N=50)**	0.86 (0.68)	1.05 (0.75)	-0.19 (0.78)	0.0869

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

4.4 Concentration of aMMP-8

The results are presented first as the total aMMP-8 (ng/ml) concentration in UPJ and LOJ at different time points. After that the aMMP-8 concentration was presented separately in UPJ and LOJ per visit.

4.4.1 Total concentration of aMMP-8 in upper and lower jaw

Before treatment with MBA (T0), the total concentration of aMMP-8 in subgingival biofilm in UPJ and LOJ was 5.29 ng/ml, which means heathy/not inflamed gingiva. On the next visit (T1), the mean value increased to 10.21 ng/ml was scored as low degree of inflammation ($p<0.05$). At T2, there was a decrease in aMMP-8 concentration to 8.55 ng/ml. In the next visit (T3) the average value increased again to 12.26 ng/ml ($p<0.05$) and then decreased at T4 to 10.43 ng/ml, but significantly increased when compared to T0 ($p<0.05$). The mean value of total aMMP-8 concentration reached its maximum peak at T5 with 13.05 ng/ml ($p<0.05$) evaluated as low degree of inflammation without risk of progressive periodontal tissue loss according to the reference values. Figure 15 illustrates the total concentration of aMMP-8 variations at different time points and Table 14 shows all the values of the total aMMP-8 concentration.

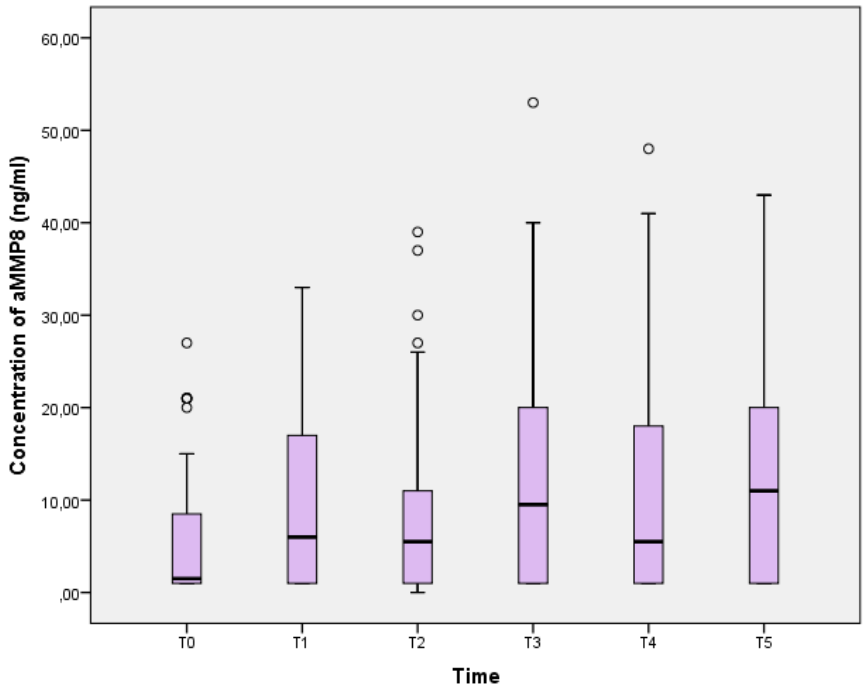


Figure 15: Boxplot showing the total aMMP-8 concentration in UPJ and LOJ at different time points.

Table 14: Statistical parameters of total aMMP-8 concentration at different time points

aMMP-8 total	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean changes from baseline (SD)	p-value
Baseline (T0) **(N=55)**	1.00	5.29 (7.08)	1.50	9.00	0 – 27	NA	NA
Time 1 (T1) **(N=53)**	1.00	10.21 (9.95)	6.00	18.00	0 – 33	4.16 (9.15)	0.0017*
Time 2 (T2) **(N=55)**	1.00	8.55 (9.54)	5.50	11.00	0 – 39	2.63 (10.45)	0.0677
Time 3 (T3) **(N=55)**	1.00	12.26 (12.8)	9.50	20.00	0 – 53	6.34 (11.87)	0.0002*
Time 4 (T4) **(N=54)**	1.00	10.43 (11.70)	5.50	18.00	0 – 48	4.42 (13.43)	0.0192*
Time 5 (T5) **(N=50)**	1.00	13.05 (11.93)	11.00	20.00	0 – 43	7.01 (12.04)	0.0001*

SD: Standard deviation; MV: mean value T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001

4.4.2 aMMP-8 – comparison between upper and lower jaw

At Baseline (T0) the aMMP-8 concentration in subgingival biofilm in UPJ was 3.03 ng/ml, considered as heathy/not inflamed gingiva. On the next visit, there was an increase statistically significant ($p<0.05$) to.5.37 ng/ml followed by a decrease at T2. Afterwards increased statistically significant ($p<0.05$) until 6.32 ng/ml at T5 (Table 15), signalizing an inflammations tendency without risk for advanced periodontal disease.

In LOJ the variations were similar to UPJ. At Baseline (T0) the aMMP-8 concentration was 2.90 ng/ml, followed by an increase statistically significant ($p<0.05$) post appliance insertion (T1) and a decrease at T2. After this time point the aMMP-8 concentration increased statistically significant ($p<0.05$) until 6.73 ng/ml at T5 (Table 16), signalizing an inflammations tendency without risk for advanced periodontal disease. There were no differences statistically significant between UPJ and LOJ (Table 17). Figure 16 shows the aMMP-8 variations at different time points.

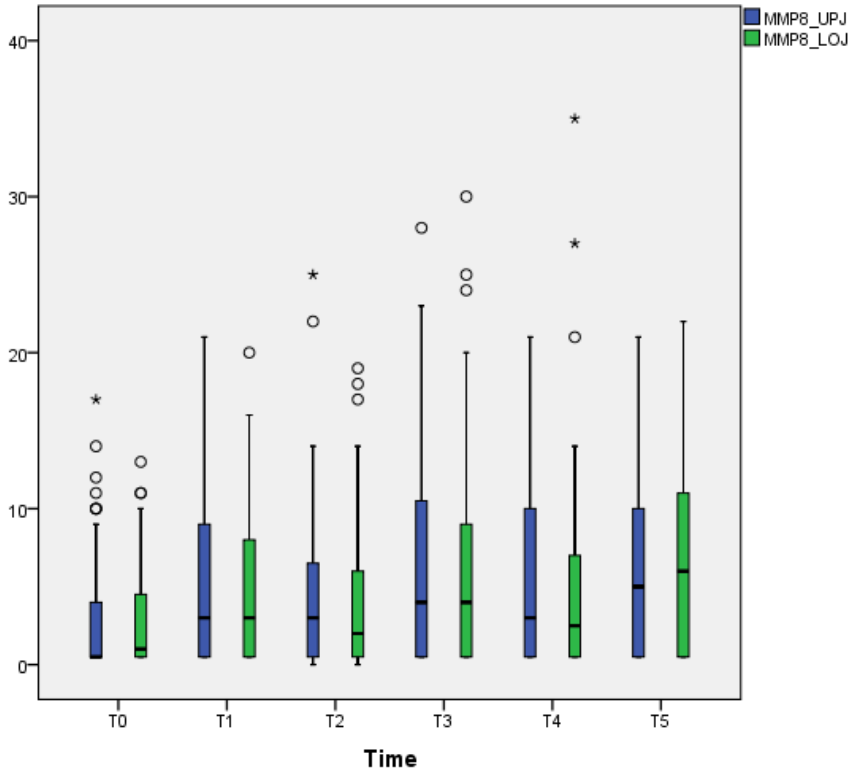


Figure 16: Boxplot diagram illustrating the aMMP-8 concentration in UPJ and LOJ separated at different time points.

Table 15: Statistical parameters of aMMP-8 concentration in UPJ at different time points

aMMP-8 – UPJ	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean change from baseline (SD)	p-value
Baseline (T0) **(N=55)**	0.50	3.03 (4.18)	0.50	4.00	1 – 17	NA	NA
Time 1 (T1) **(N=53)**	0.50	5.37 (5.72)	3.00	9.50	1 – 21	2.27 (5.89)	0.0069*
Time 2 (T2) **(N=55)**	0.50	4.33 (5.20)	3.00	7.00	0 – 25	1.30 (5.90)	0.1079
Time 3 (T3) **(N=55)**	0.50	6.35 (7.19)	4.00	11.00	1 – 28	3.33 (7.43)	0.0016*
Time 4 (T4) **(N=54)**	0.50	5.32 (6.14)	3.00	10.00	1 – 21	2.25 (7.36)	0.0289*
Time 5 (T5) **(N=50)**	0.50	6.32 (6.09)	5.00	10.25	1 – 21	3.26 (6.61)	0.0010*

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001

Table 16: Statistical parameters of aMMP-8 concentration in LOJ at different time points

aMMP-8 – LOJ	Lower Quartile	MV (SD)	Median	Upper Quartile	Min.-Max.	Mean change from baseline (SD)	p-value
Baseline (T0) **(N=55)**	0.50	2.90 (3.42)	1.00	5.00	1 – 13	NA	NA
Time 1 (T1) **(N=53)**	0.50	4.85 (5.09)	3.00	8.00	1 – 20	1.89 (4.42)	0.0030*
Time 2 (T2) **(N=55)**	0.50	4.23 (5.06)	2.00	6.00	0 – 19	1.33 (5.32)	0.0696
Time 3 (T3) **(N=55)**	0.50	5.91 (6.81)	4.00	9.00	1 – 30	3.01 (6.15)	0.0006*
Time 4 (T4) **(N=54)**	0.50	5.11 (6.94)	2.50	7.00	1 – 35	2.17 (7.54)	0.0394*
Time 5 (T5) **(N=50)**	0.50	6.73 (6.58)	6.00	11.00	1 – 22	3.75 (6.37)	0.0001*

SD: Standard deviation; MV: mean value; T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001

Table 17: aMMP-8 – Comparison between UPJ and LOJ

aMMP-8 – UPJ	**aMMP-8** MV (SD)	**aMMP-8** MV (SD)	**aMMP-8** MV (SD)	p-value
Baseline (T0) **(N=55)**	3.03 (4.18)	2.90 (3.42)	0.13 (2.84)	0.7409
Time 1 (T1) **(N=53)**	5.37 (5.72)	4.85 (5.09)	0.52 (4.28)	0.3814
Time 2 (T2) **(N=55)**	4.33 (5.21)	4.23 (5.06)	0.10 (3.76)	0.8445
Time 3 (T3) **(N=55)**	6.35 (7.19)	5.91 (6.81)	0.45 (5.67)	0.5623
Time 4 (T4) **(N=54)**	5.32 (6.14)	5.11 (6.94)	0.21 (5.89)	0.7916
Time 5 (T5) **(N=50)**	6.32 (6.10)	6.73 (6.58)	-0.41 (4.32)	0.5048

SD: Standard deviation; MV: mean value T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

4.5 Microbial analysis

There was a statistically significant increase ($p<0.05$) in the frequency of *Tf*, *Fs*, *Cr*, *Cs*, and *Ec* after appliance placement. For the other investigated bacteria (*Aa*, *Pg*, *Td*, *Pi*, *Pm*, *En*) there was an increased but not statistically significant.

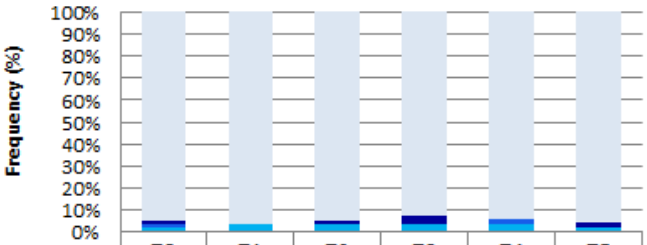
In the case of one subject, the microbial analysis at T5 is missing because there was a technical problem in the laboratory with his sample.

The results are presented as the frequency (percentage) of patients positive to each PP at different time points (T0, T1, T2, T3, T4, and T5) in UPJ and LOJ.

4.5.1 *Aggregatibacter actinomycetemcomitans*

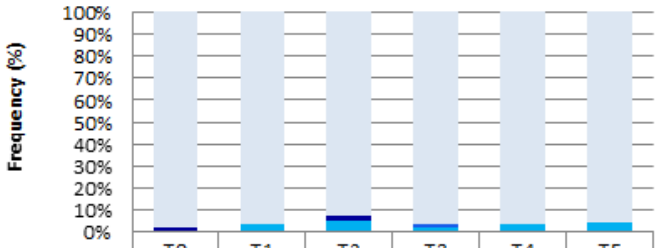
Frequency alterations of *Aa* during the observed period were not significant. In UPJ the maximum peak occurred at T3 with 7.3% of the participants showing positives finding to *Aa*. In LOJ the maximum peak occurred at T2 (7.3%), declining at T3 and remaining constant until T5 (4.1%). Tables 18 and 19 show the percentages of subjects with PP counts and Figure 17 illustrates the variation of *Aa* in UPJ and LOJ at different time points. The variations of *Aa* frequency was not significant (Tables 20, 21).

***Aggregatibacter actinomycetemcomitans* - Upper Jaw**



	T0	T1	T2	T3	T4	T5
not verified	52	51	52	51	51	47
extremely increased	1	0	1	2	0	1
highly increased	1	0	0	0	1	0
increased	1	2	2	2	2	1

***Aggregatibacter actinomycetemcomitans* - Lower Jaw**



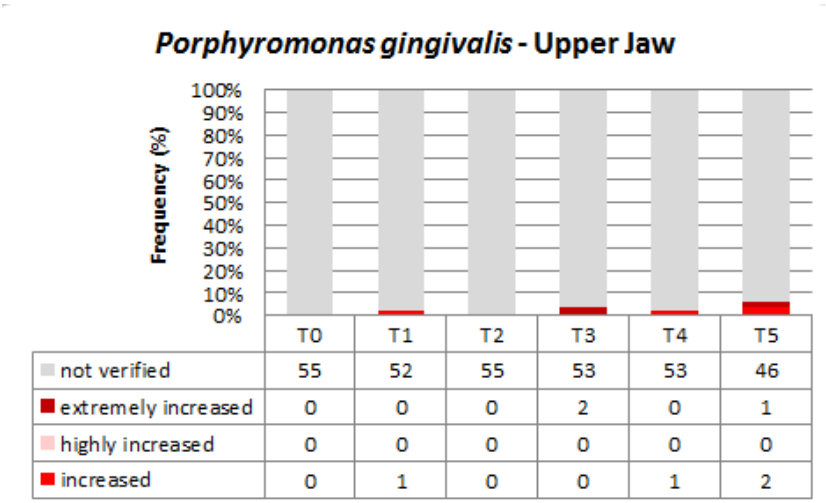
	T0	T1	T2	T3	T4	T5
not verified	54	51	51	53	52	47
extremely increased	1	0	1	0	0	0
highly increased	0	0	0	1	0	0
increased	0	2	3	1	2	2

T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

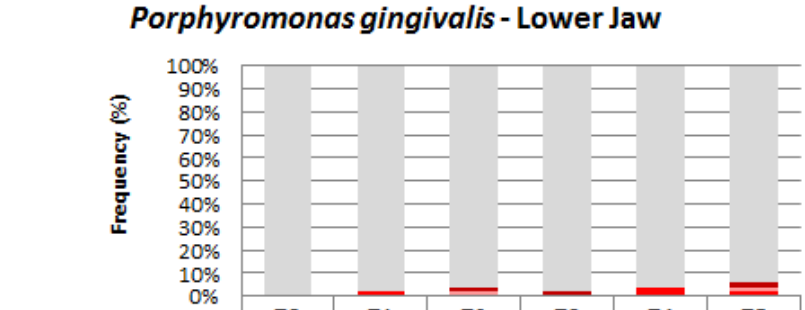
Figure 17: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Aggregatibacter actinomycetemcomitans* in UPJ and LOJ at different time points.

4.5.2 Red complex

As shown in Figure 18, *Pg* was not detected in any subject at T0. At the end of the observed period, 6% of the patients showed positive sites to *Pg* in both UPJ and LOJ, although these variations were not significant (Tables 20 and 21). Tables 18 and 19 show the percentages of subjects with PP counts.



	T0	T1	T2	T3	T4	T5
not verified	55	52	55	53	53	46
extremely increased	0	0	0	2	0	1
highly increased	0	0	0	0	0	0
increased	0	1	0	0	1	2



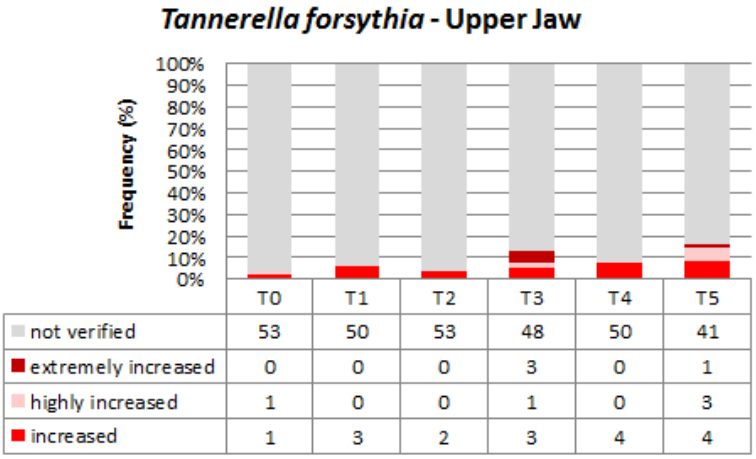
	T0	T1	T2	T3	T4	T5
not verified	55	52	53	54	52	46
extremely increased	0	0	1	1	0	1
highly increased	0	0	1	0	0	1
increased	0	1	0	0	2	1

T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

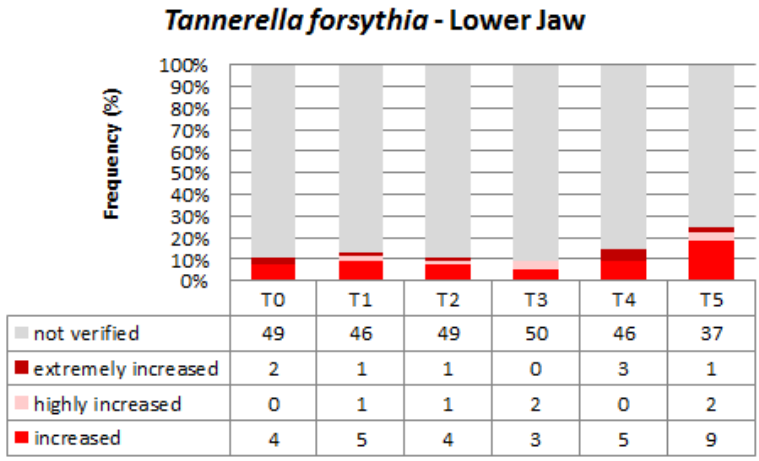
Figure 18: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Porphyromonas gingivalis* in UPJ and LOJ at different time points.

Tf is one of the 11 evaluated PP that significantly increased during the observed period. In UPJ, *Tf* significantly increased from T0 (3.6%) to T5 (16.3%) ($p<0.05$), with peaks at T3 (12.8%), 3 months after appliance insertion and at T5.

In LOJ, *Tf* also significantly increased in a time-response manner from T0 (10.9%) to T5 (24.5%) ($p<0.05$). Figure 19 illustrates the frequency of *Tf* in UPJ and LOJ and table 18 shows the percentages of subjects with PP counts at different time points for UPJ and table 19 shows the percentages for LOJ.



	T0	T1	T2	T3	T4	T5
not verified	53	50	53	48	50	41
extremely increased	0	0	0	3	0	1
highly increased	1	0	0	1	0	3
increased	1	3	2	3	4	4



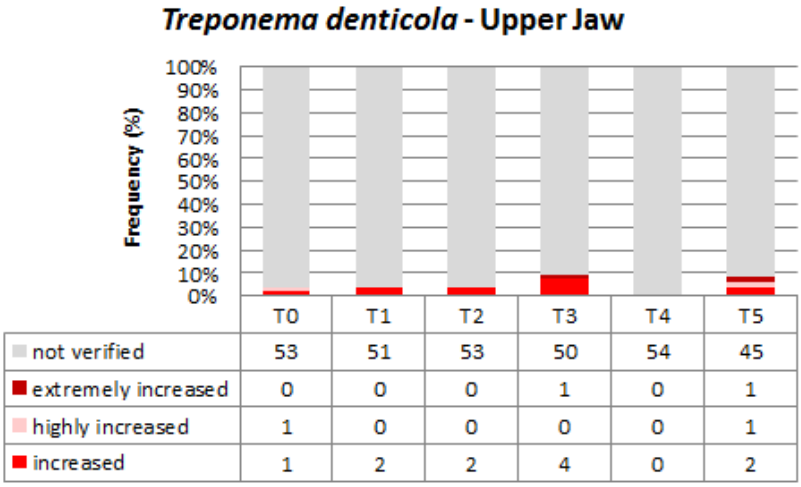
	T0	T1	T2	T3	T4	T5
not verified	49	46	49	50	46	37
extremely increased	2	1	1	0	3	1
highly increased	0	1	1	2	0	2
increased	4	5	4	3	5	9

T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

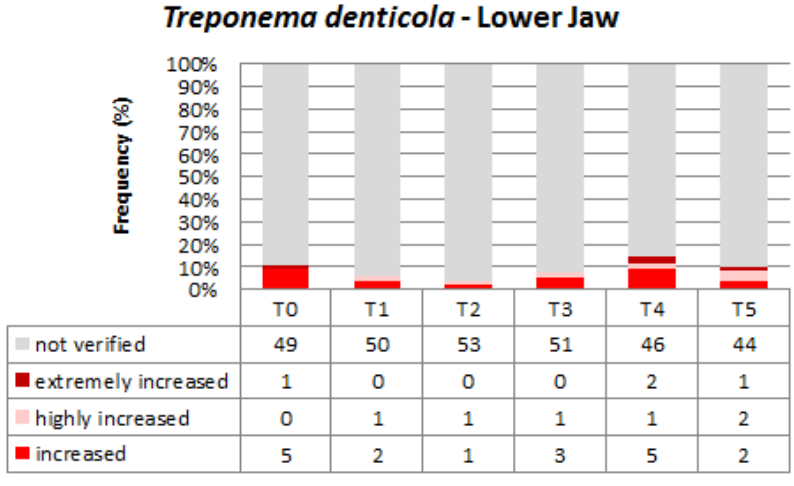
Figure 19: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Tannerella forsythia* in UPJ and LOJ at different time points.

In UPJ, *Td* frequency remains constant until T2 (3.6%), followed by an increase at T3 (9.1%). At T4 *Td* was not detected at any subject. Then occurred a small increase at T5 (8.1%), but the changes were not statistically significant (Table 18).

The frequency of subjects with *Td* in LOJ at T0 was 10.9%. At T1 and T2 the frequency decreased, increasing until T4 (14.9%), when reached its maximum peak. After that, the frequency at T5 (9.1%) decreased again (Table 19). The variations in UPJ and LOJ were not statistically significant (Figure 20, Tables 20 and 21).



	T0	T1	T2	T3	T4	T5
not verified	53	51	53	50	54	45
extremely increased	0	0	0	1	0	1
highly increased	1	0	0	0	0	1
increased	1	2	2	4	0	2



	T0	T1	T2	T3	T4	T5
not verified	49	50	53	51	46	44
extremely increased	1	0	0	0	2	1
highly increased	0	1	1	1	1	2
increased	5	2	1	3	5	2

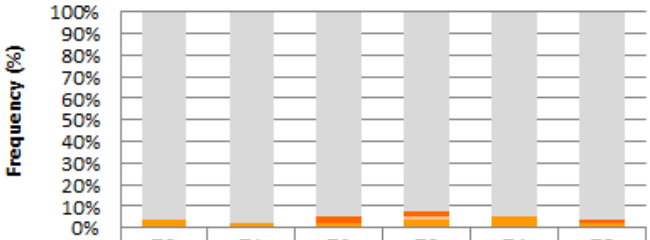
T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

Figure 20: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Treponema denticola* in UPJ and LOJ at different time points.

4.5.3 Orange complex

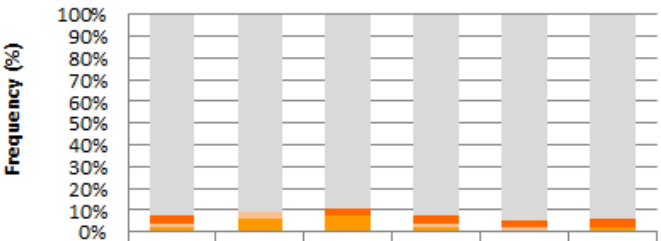
The percentage of patients positive to *Pi* in UPJ was 3.6% at T0. At T1 just one subject presented *Pi*. Afterwards, *Pi* gradually increased until T3 (7.2%), reaching its maximum peak. Later, *Pi* gradually declined until T5 (2%), reaching almost the same frequency as at T0 (Table 18). In LOJ, the total percentage of individuals positive to *Pi* was slightly higher. It ascended gradually, reaching its maximum peak at T2 (10.9%) and, then, it gradually declined to T4 and remained constant until T5 (6.1%) (Table 19, Figure 21). Variations at UPJ and LOJ were not statistically significant (Tables 20 and 21).

***Prevotella intermedia* - Upper Jaw**



	T0	T1	T2	T3	T4	T5
not verified	53	52	52	51	51	47
extremely increased	0	0	2	1	0	1
highly increased	0	0	0	1	0	0
increased	2	1	1	2	3	1

***Prevotella intermedia* - Lower Jaw**



	T0	T1	T2	T3	T4	T5
not verified	51	48	49	51	51	46
extremely increased	2	0	2	2	2	2
highly increased	1	2	0	1	1	0
increased	1	3	4	1	0	1

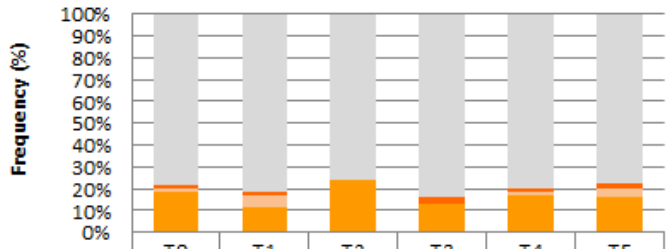
T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

Figure 21: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Prevotella intermedia* in UPJ and LOJ at different time points.

The number of individuals positive to *Pm* in UPJ remained basically the same at all time points. There was a small reduction at T3 (16.3%) and after T4 there was an increase until the end of the evaluated period (22.4%) (Table 18).

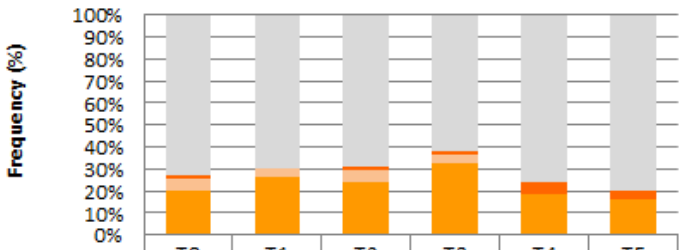
In LOJ, the percentage of individuals with positive findings to *Pm* was slightly higher in all periods. It ascended gradually (27.3%), reaching its maximum peak at T3 (38.1%) and, then, declined gradually until T5 (20.4%) (Table 19). Figure 22 illustrates the frequency of *Pm* in UPJ and LOJ. In both UPJ and LOJ, the variations were not statistically significant (Tables 20 and 21).

***Parvimonas micra* - Upper Jaw**



	T0	T1	T2	T3	T4	T5
not verified	43	43	42	46	43	38
extremely increased	1	1	0	2	1	1
highly increased	1	3	0	0	1	2
increased	10	6	13	7	9	8

***Parvimonas micra* - Lower Jaw**

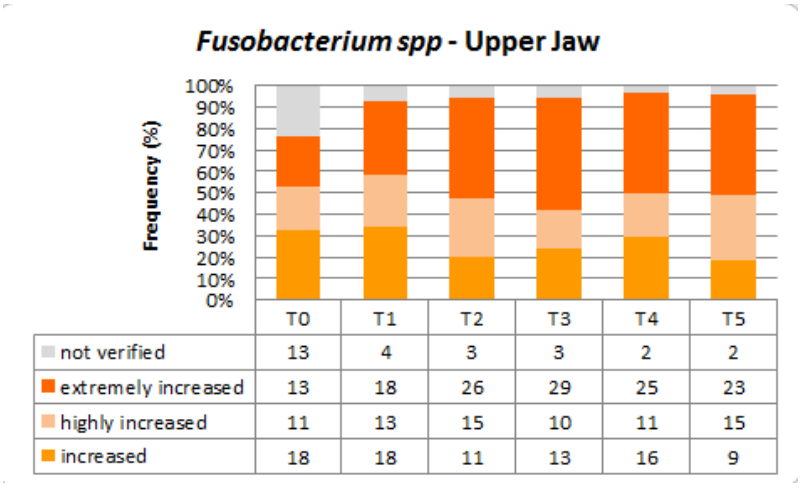


	T0	T1	T2	T3	T4	T5
not verified	40	37	38	34	41	39
extremely increased	1	0	1	1	3	2
highly increased	3	2	3	2	0	0
increased	11	14	13	18	10	8

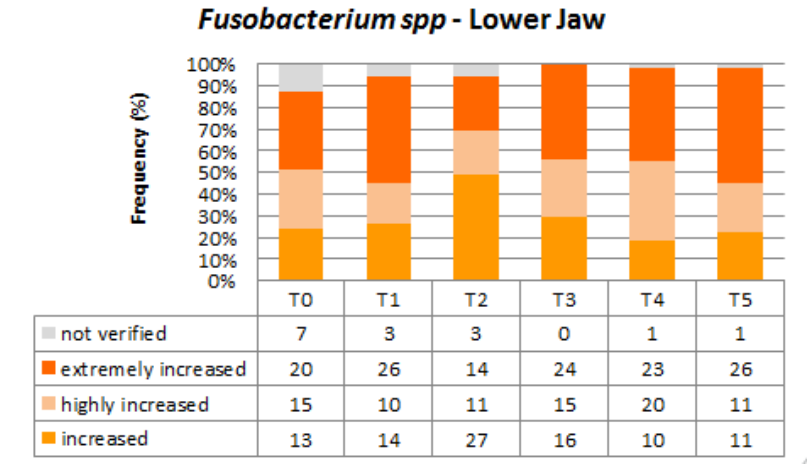
T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

Figure 22: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Parvimonas micra* in UPJ and LOJ at different time points.

The number of subjects with positive findings to *Fs* in UPJ significant increased from the first recording (T0) until T5 ($p<0.05$). In UPJ, 76.3% of the patients demonstrated positive sites to *Fs* at T0. This number continually increased showing a maximum of 96.3% of the subjects positive to *Fs* at T4 (Table 18, 20). In LOJ, 87.3% of the patients showed positive findings to *Fs* and this number increased continuously until T3 when 100% of the individuals presented this microorganism. At T4 there was a small decline followed by a significant increased ($p<0.05$) at T5, when T5 97.9% presented *Fs* (Table 19, 21). Figure 23 illustrates the frequency of *Fs* in UPJ and LOJ at different time points.



	T0	T1	T2	T3	T4	T5
not verified	13	4	3	3	2	2
extremely increased	13	18	26	29	25	23
highly increased	11	13	15	10	11	15
increased	18	18	11	13	16	9



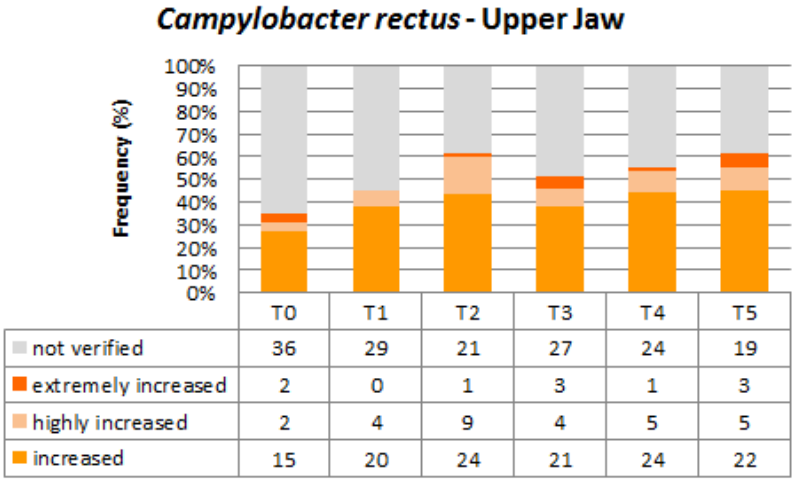
	T0	T1	T2	T3	T4	T5
not verified	7	3	3	0	1	1
extremely increased	20	26	14	24	23	26
highly increased	15	10	11	15	20	11
increased	13	14	27	16	10	11

T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

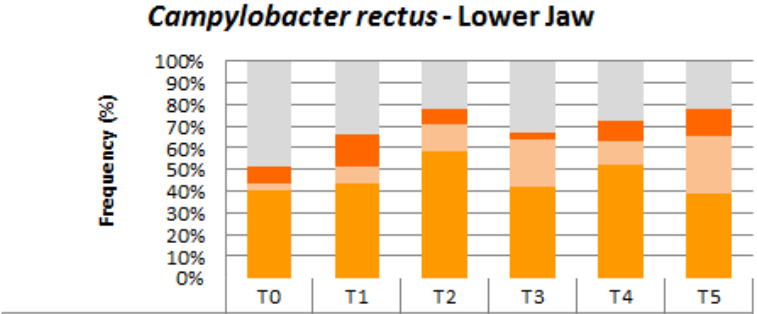
Figure 23: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Fusobacterium spp* in UPJ and LOJ at different time points.

4.5.3.1 Bacteria associated with the Orange Complex

The number of subjects with positive findings to *Cr* in UPJ was 34.5% at T0. The number of subjects significantly increased ($p<0.05$) until T2. At T5 occurred a significant increase ($p<0.05$) as 61.2% of the total subjects presented *Cr* in the microbiological analyses (Tables 18, 20). The patterns of LOJ changes were the same as in the maxilla and all changes were statistically significant when compared to T0 (Tables 19, 21). Figure 24 illustrates the frequency of *Cr* in UPJ and LOJ at different time points.



	T0	T1	T2	T3	T4	T5
not verified	36	29	21	27	24	19
extremely increased	2	0	1	3	1	3
highly increased	2	4	9	4	5	5
increased	15	20	24	21	24	22

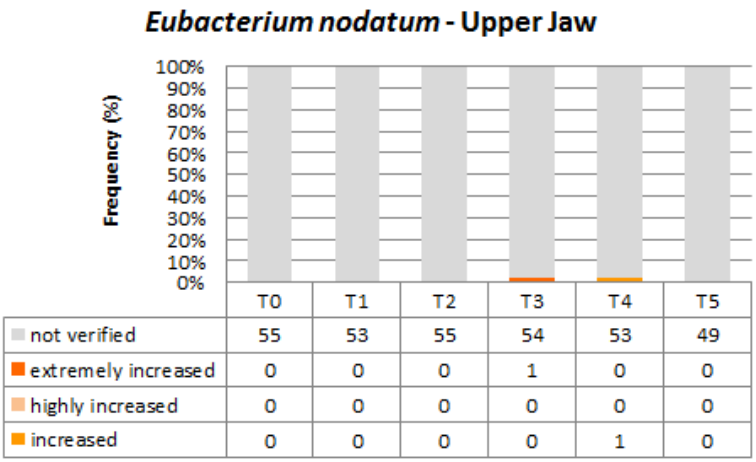


	T0	T1	T2	T3	T4	T5
not verified	27	18	12	18	15	11
extremely increased	4	8	4	2	5	6
highly increased	2	4	7	12	6	13
increased	22	23	32	23	28	19

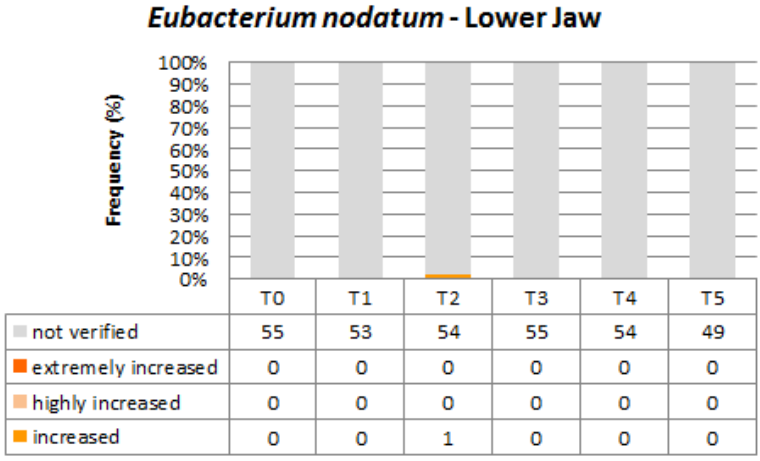
T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

Figure 24: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Campylobacter rectus* in UPJ and LOJ at different time points.

En was found in only three patients during the study. In UPJ, *En* was found once at T3 and one time at T4. In LOJ, *En* was isolated in only one patient at T2. Figure 25 demonstrates the frequency of *En* in UPJ and LOJ at different time points.



	T0	T1	T2	T3	T4	T5
not verified	55	53	55	54	53	49
extremely increased	0	0	0	1	0	0
highly increased	0	0	0	0	0	0
increased	0	0	0	0	1	0



	T0	T1	T2	T3	T4	T5
not verified	55	53	54	55	54	49
extremely increased	0	0	0	0	0	0
highly increased	0	0	0	0	0	0
increased	0	0	1	0	0	0

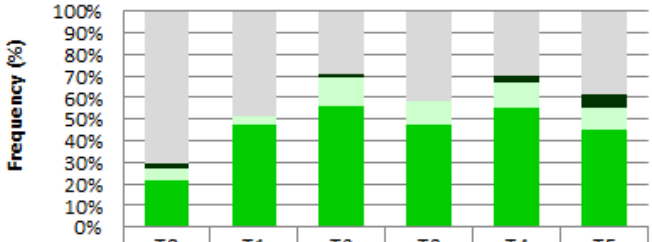
T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

Figure 25: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Eubacterium nodatum* in UPJ and LOJ at different time points.

4.5.4 Green complex

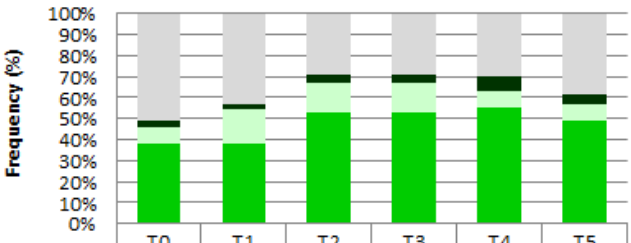
Thirty percent of the patients presented the pathogenic bacteria *Ec* in UPJ at T0. The number of subjects with positive findings to *Ec* increased significantly ($p<0.05$) until T2. At T3 there was a slight decrease but increased again at T4 (70.4%). At T5 (61.8%) the number decreased and all the variations were statistically significant when compared to T0 ($p<0.05$) (Tables 18, 20). In LOJ there was a continuous increase of patients positive to *Ec* until T2. It remained the same at T3 (72.3%) and reduced at T5. Variations between T0 vs T2; T0 vs T3 and T0 vs T4 were statistically significant ($p<0.05$) (Tables 19, 21). Figure 26 illustrates the frequency of *Ec* in UPJ and LOJ at different time points.

***Eikenella corrodens* - Upper Jaw**



	T0	T1	T2	T3	T4	T5
not verified	39	26	16	23	16	19
extremely increased	1	0	1	0	2	3
highly increased	3	2	7	6	6	5
increased	12	25	31	26	30	22

***Eikenella corrodens* - Lower Jaw**

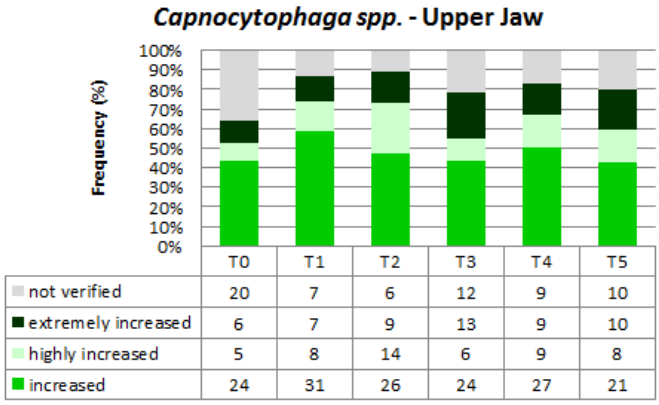


	T0	T1	T2	T3	T4	T5
not verified	28	23	16	16	16	19
extremely increased	2	1	2	2	4	2
highly increased	4	9	8	8	4	4
increased	21	20	29	29	30	24

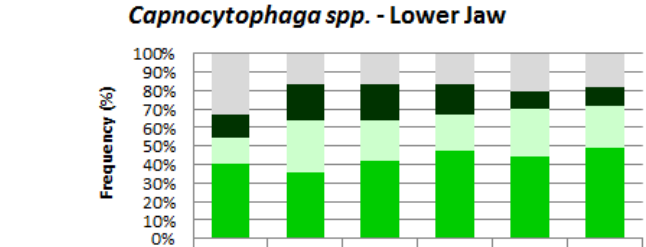
T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA.

Figure 26: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Eikenella corrodens* in UPJ and LOJ at different time points.

The PP *Cs* was found in 62.9% of patients in UPJ at T0. This number increased significantly ($p<0.05$) until T4 (84.4%). At T5 the frequency of *Cs* decreased and 79.6% of the total subjects presented *Cs* in microbiological analyses. All the variations were statistically significant when compared to T0 ($p<0.05$) (Tables 18, 20). In LOJ it was observed a statistically significant ($p<0.05$) increase in the number of positive patients to Cs at T1 (83%) as compared to T0 (67.2%). At T2 and T3 the percentage remained constant and a slight reduction was detected at T4 (79.6%). Afterwards, there was an increase to 81.6% at T5. The increases at T1, T2 and T3 were considered significant when compared to T0 ($p<0.05$) (Tables 19, 21). Figure 27 shows the frequency of *Cs* in UPJ and LOJ at different time points.



	T0	T1	T2	T3	T4	T5
not verified	20	7	6	12	9	10
extremely increased	6	7	9	13	9	10
highly increased	5	8	14	6	9	8
increased	24	31	26	24	27	21



	T0	T1	T2	T3	T4	T5
not verified	18	9	9	9	11	9
extremely increased	7	10	11	9	5	5
highly increased	8	15	12	11	14	11
increased	22	19	23	26	24	24

T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA

Figure 27: Bar diagram showing the frequency (%) and table showing the number of patients with positive and negative results for *Capnocytophaga spp* in UPJ and LOJ at different time points.

Table 18: Percentages of subjects with PP counts in UPJ at different time points

PP – UPJ	% T0	% T1	% T2	% T3	% T4	% T5
Ag	5.5	3.8	5.5	7.3	5.6	4.1
Pg	0	1.9	0	3.6	1.9	6.1
Tf	3.6	5.7	3.6	12.8	7.4	16.3*
Td	3.6	3.8	3.6	9.1	0	8.1
Pi	3.6	1.9	5.4	7.2	5.6	4.1
Pm	21,8	18.9	23.6	16.4	20.5	22.4
Fs	76.3	92.5*	94.5*	94.5*	96.3*	95.9*
Cr	34.5	45.2	61.8*	51	55.6	61.2*
En	0	0	0	1.8	1.9	0
Ec	29.1	51*	70.9**	58.2*	70.4**	61.2*
Cs	62.9	86.8*	89.2*	78.1*	83.4*	79.6*
	(N=55)	(N=53)	(N=55)	(N=55)	(N=54)	(N=49)

T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001

Table 19: Percentages of subjects with PP counts in LOJ at different time points

PP – LOJ	% T0	% T1	% T2	% T3	% T4	% T5
Ag	1.8	3.8	7.3	3.6	3.7	4.1
Pg	0	1.9	3.6	1.8	3.7	6
Tf	10.9	13.2	10.9	9.1	14.9	24.5*
Td	10.9	5.7	3.6	7.3	14.9	10.2
Pi	7.2	9.5	10.9	7.2	5.6	6
Pm	27.3	30.2	30.9	38.1	24.1	20.4
Fs	87.3	94.4	94.5	100	98.1	98*
Cr	50.9	66*	78.2*	67.2	72.2*	77.5*
En	0	0	1.8	0	0	0
Ec	49.1	56.6	70.8*	70.8*	70.4*	61.3
Cs	67.2	83*	83.6*	83.7*	79.6	81.6
	(N=55)	(N=53)	(N=55)	(N=55)	(N=54)	(N=49)

T0: Baseline: 1 week before MBA; T1: 3 weeks after MBA; T2: 6 weeks after MBA; T3: 3 months after MBA; T4: 6 months after MBA; T5: 1 year after MBA. * p<0.05; ** p<0.0001

The comparison of PP at T0 to T1, T2, T3, T4, and T5 are demonstrated in Table 20 for UPJ and Table 21 for LOJ. Then the frequency of the microorganisms in UPJ and LOJ is summarized in Figures 28 and 29 at different time points.

Table 20: Comparison of PP at T0 to T1, T2, T2, T4, and T5 in UPJ

PP	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5
Aa_UPJ	1.0000	0.0832	0.0917	1.0000	1.0000
Pg_ UPJ	0.3219	–	0.1592	0.3219	0.1030
Tf_ UPJ	0.6590	0.8296	0.0770	0.5823	0.0151*
Td_ UPJ	0.7845	0.8296	0.2262	0.1676	0.0546
Pi_ UPJ	0. 3219	0.2606	0.1963	0.5686	0.7096
Pm_ UPJ	0.7551	0.9185	0.6087	0.7956	0.6843
Fs_ UPJ	0.0365*	0.0001*	0.0004*	0.0003*	0.0002*
Cr_ UPJ	0.4847	0.0030*	0.0917	0.0588	0.0121*
En_ UPJ	–	–	0.3218	0.3219	–
Ec_ UPJ	0.0453*	<.0001**	0.0039*	<.0001**	0.0022*
Cs_ UPJ	0.0064*	0.0002*	0.0032*	0.0137*	0.0423*

*p <0.05; ** p<0.0001; null cells indicate that no subjects had the bacteria.

Table 21: Comparison of PP at T0 to T1, T2, T2, T4, and T5 in LOJ

PP	T0 vs T1	T0 vs T2	T0 vs T3	T0 vs T4	T0 vs T5
Aa_LOJ	1.0000	0.0832	0.8547	1.0000	1.0000
Pg_ LOJ	0.3219	0.1635	0.3218	0.1592	0.0950
Tf_ LOJ	0.8296	0.9081	0.5214	0.4105	0.0152*
Td_ LOJ	0.0513	0.1404	0.2285	0.2784	0.2206
Pi_ LOJ	0.7423	0.6589	1.0000	0.7489	0.6728
Pm_ LOJ	0.9027	0.7251	0.3374	0.7788	0.8278
Fs_ LOJ	0.2264	0.0682	0.0721	0.0570	0.0127*
Cr_ LOJ	0.0243*	0.0037*	0.0816	0.0467*	0.0009*
En_ LOJ	–	0.3218	–	–	–
Ec_ LOJ	0.4297	0.0120*	0.0284*	0.0361*	0.2572
Cs_ LOJ	0.0093*	0.0207*	0.0436*	0.2693	0.4725

*p <0.05; null cells indicate that no subjects had the bacteria.

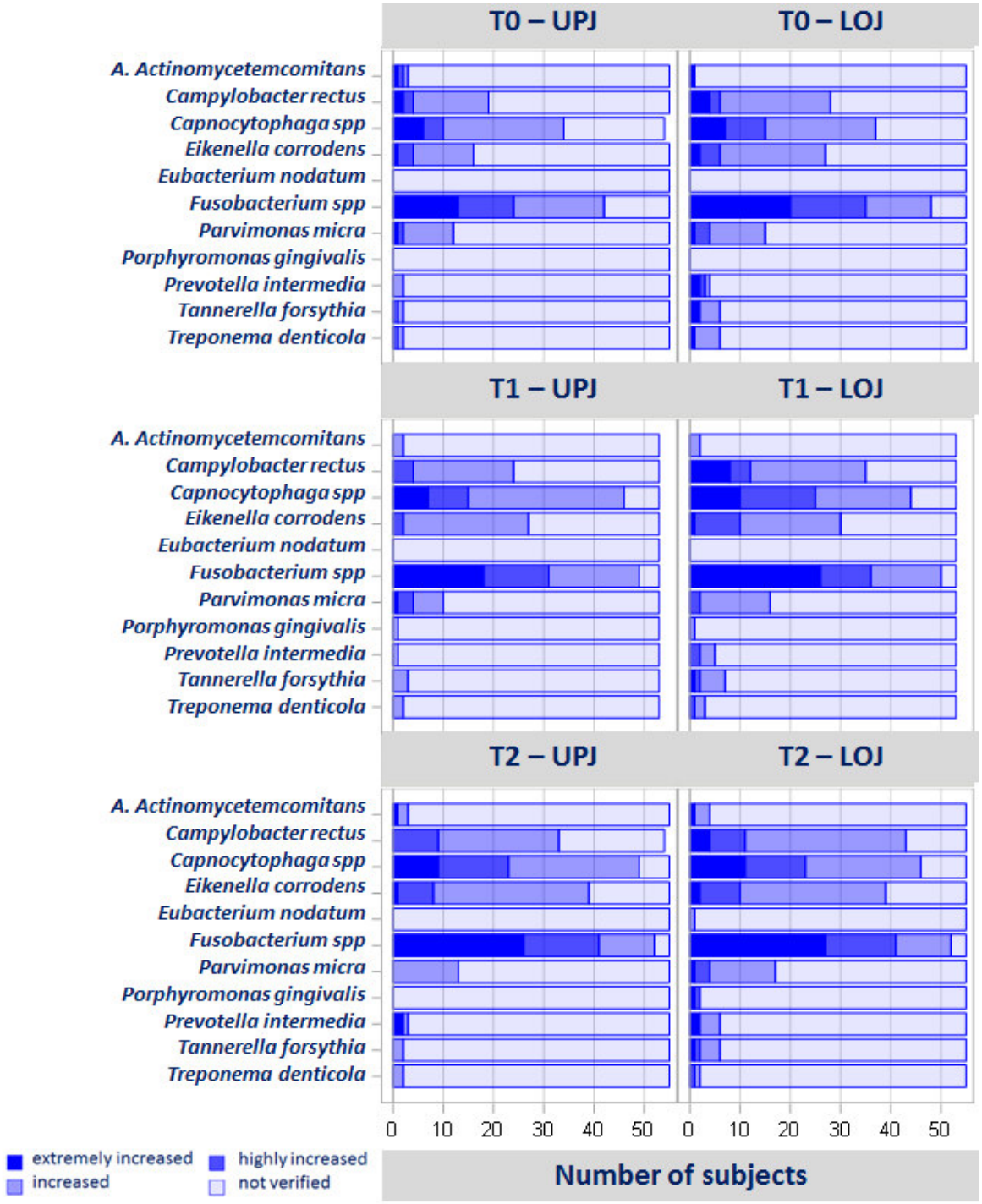


Figure 28: Bar diagram showing the level of all eleven periodontal pathogen in upper and lower jaw at T0,T1,T2.

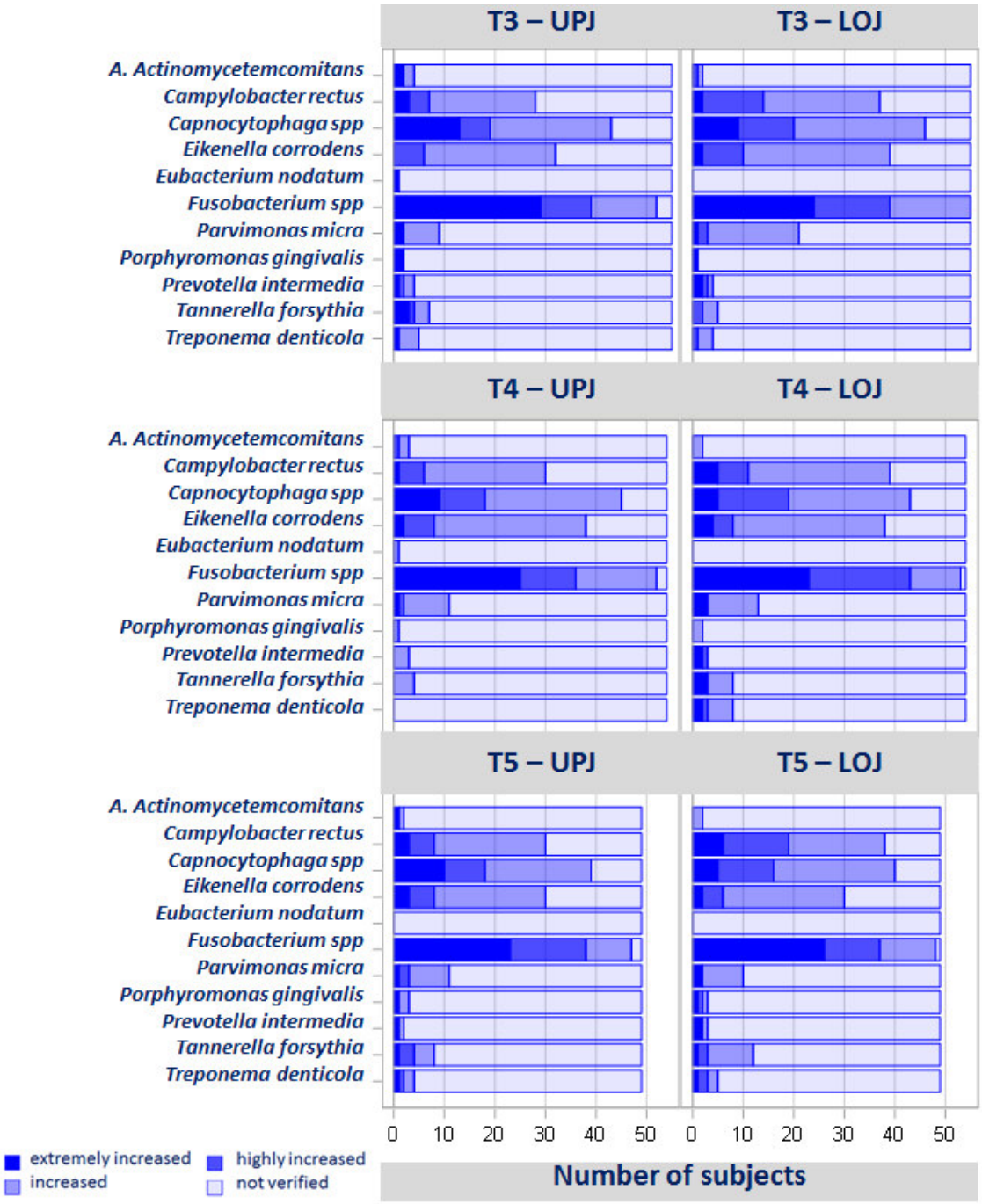


Figure 29: Bar diagram showing the level of all eleven periodontal pathogen in upper and lower jaw at T3,T4,T5.

5. Discussion

Periodontal inflammation and caries are the main concern during fixed appliance treatment. Several factors may affect periodontal heath and microbial population during orthodontic treatment, such as brackets and arch wires presence, plaque accumulation, hormonal levels and subgingival microbiota changes during puberty, host immunity, and patient compliance (154-157).

5.1 Clinical Indices

The present study reports a significant increase in GI and PI values in adolescents after MBA insertion. Total GI and GI in UPJ significant increased after 6 weeks ($p<0.05$) and 3 months ($p<0.0001$) of brackets placement until the last time point, 1 year later. In LOJ a continuous increased was observed at 6 weeks ($p<0.05$) of treatment until the observed period end ($p<0.0001$), when it reached its peak, signalizing gingival inflammation presence. Therefore, our results provide original evidence that fixed orthodontic appliance treatment can negatively influence gingival health even up to 1 year after treatment initiation.

Inadequate oral hygiene around brackets may result in gingival hyperplasia which can progress to periodontitis, if a slight inflammation is not controlled and plaque accumulation continues for a long time (158).

Concerning the total PI values, it continually increased until 3 months after bracket placement ($p<0.05$), when it reached its maximum peak. After 6 months of therapy there was a decreased in PI values, followed by an increase at the last time point ($p<0.05$). The same variation was observed in UPJ and LOJ.

Increase in PI values in the first therapy months may be due to patient's unfamiliarity with the appliance and difficulty in maintaining a proper oral hygiene. The reduction noted in PI values after 6 months of treatment supports the fact that dental alignment allows the patient to achieve a good gingival condition around previously misaligned teeth (33, 159). However, the long orthodontic treatment duration can lead to a lack of patient motivation to perform good oral hygiene, justifying the observed increase in PI values 1 year post fixed appliance insertion.

Our results were in accordance with Naranjo et al. (26) and Guo et al. (11), regarding the elevated GI and PI values 3 months after MBA insertion. Ristic et al. (31, 32) also reported an increase after 3 months of therapy followed by a decrease 6 months later. Liu et al. (122) reported a significant decrease of these indices after 1 week of appliance removal, returning to pre-treatment values. Kim et al. (30) also related a decrease in GI and PI values 6 months after therapy end. A systematic review conducted by Cerroni et al. suggests that there is moderate scientific evidence that a fixed appliance negatively influences periodontal status (160). On the other hand, Gomes and co-workers (161) stated that orthodontic appliances use is not necessarily related to periodontal conditions aggravation, but rather to each person susceptibility to periodontal disease.

Nonetheless, the majority published studies in the literature have a patient follow-up up to 3 or 6 months after brackets placement or post appliance removal and with a small sample size. In contrast, our study accompanied 55 patients until 6 months and 50 subjects up to 1 year of MBA treatment. More long-term studies are necessary to be conduct on a wider sample size containing a control group to evaluate MBA effects on periodontium after years of treatment.

Hence, our findings reject the null hypothesis and endorse previous reports showing an existing correlation between GI and PI values before and during orthodontic treatment. Accordingly, it can be suggested that plaque accumulation favored by brackets and arch wires can cause gingival inflammation. Moreover, it is implied that MBA induces gingival inflammation without damaging the dental support tissues.

Though, a present study limitation is regarding to GI and PI. Both indices express buccal and palatinal/lingual surfaces sum values. It would be interesting to compare these two surfaces, once the brackets were bonded on the buccal teeth surface. A second point which should be considered is GI and PI distribution between anterior and posterior segments, and if there are differences between right and left sides.

5.2 Subgingival Samples – aMMP-8

PD diagnosis has been based mainly on clinical and radiographic periodontal tissues assessment. Conventional parameters may fail to identify PD specific active sites and its manifestations, resulting in subsequent supporting tissue loss. GCF is a serum exudate that is collected in gingival margin or within gingival crevice and, contains high levels of a vast range of biochemical factors offering an adequate disease activity diagnosis. GCF is an attractive oral fluid due to its facility of sampling and the possibility to collect samples from multiple sites within oral cavity simultaneously (108, 162). A range of biomarkers implicated in the development and progression of PD have been investigated, such as cytokines (interleukins) and MMPs (104, 105, 163-165).

MMP-8 concentration assessment in GCF has proven to be an efficient method for early diagnosis and assessment of periodontal inflammation before permanent damage occur. It is non-invasive, avoids radiographic exposure and, is more objective than traditional methods (37, 106, 166). Although a very accurate analysis of MMP-8 concentration is only possible at one laboratory, this objective method is particularly suitable for studies in which inflammatory course processes must be documented. Therefore it was used in the present study.

Majority of studies found in the literature associate the high presence of MMP-8 in GCF of orthodontic patients with periodontal ligament remodeling process (38, 39, 167-170) and with pain mentioned by some patients during the first hours/days after appliance placement (171-173).

Surlin et al. (174) reported an increase of MMP-8 concentration in the first 4-8 hour after orthodontic appliance placement followed by a decrease to initial levels. Some subjects developed gingival overgrowth (GO) during orthodontic treatment even in bacterial plaque absence. Interestingly, in these patients MMP-8 levels continued to increase until GO appearance. Furthermore, some patients presented GO in combination with inflammation and in these cases, MMP-8 concentration was higher than in GO cases

without inflammation. In this way, the authors suggest that MMP-8 may be a possible biomarker for GO beginning.

Our study novelty was the use of aMMP-8 as periodontal biomarker in patients undergoing orthodontic treatment. Our study provides original evidence that 3 weeks after brackets placement there was a significant increase of aMMP-8 levels, which remained elevated even 1 year after treatment begin, suggesting an inflammations tendency. aMMP8 high rates evidenced in this study agree with the high GI and PI scores and elevated some PP level. Hence, our findings reject the null hypothesis that there is no correlation between the pro-inflammatory biomarker MMP-8 in the GCF before and during orthodontic treatment.

5.3 Subgingival Samples – Periodontopathogens

Fixed orthodontic appliances can contribute to plaque accumulation, which is the crucial etiological factor of PD. Supragingival biofilm accumulation may have an impact on the subgingival microbiota composition. Teza et al. concluded in their study that a high PP presence in supragingival biofilm may result in a high PP presence in subgingival biofilm (156).

The frequency of 11 PP (*Aa*, *Pg*, *Tf*, *Td*, *Fs*, *Pm*, *Pi*, *Cr*, *En*, *Ec*, *Cs*) associated with PD was examined at six different time points during MBA treatment. As shown in Tables 18 and 19, *Pm*, *Fs*, *Cr*, *Ec* and *Cs* were frequently detected pretreatment at T0 in both UPJ and LOJ, whereas *Aa*, *Tf*, *Td*, *Pi* were scarcely detected. *Pg* and *En* were not found at any subject at treatment begin.

5.3.1 *Aggregatibacter actinomycetemcomitans*

Our data showed a very low level of *Aa* without significant variations during the observed period. These results are consistent with previous studies (Ristic et al. (31, 32), Thornberg et al. (33), Choi et al. (118) and Kim et al. (119)), who also reported low frequency of *Aa* during the treatment. Ristic et al. (31) reported *Aa* presence isolated only in one subject, 1 and 3 months after bracket insertion. Moreover, Thornberg and collaborators (33) found small *Aa* frequency during 12 months of MBA treatment, followed by a decrease to null value 3 months after appliance removal. Choi et al. (118) also related only 5.8% subjects with positive findings of *Aa*, two weeks before appliance removal, followed by a decrease to 3.3% after its removal. Kim et al. (119) found *Aa* in less than 5% of the subjects during the entire orthodontic treatment.

5.3.2 Red complex

Red complex bacteria are considered very pathogenic and lead to intensive tissue destruction. They occur predominantly by advanced and aggressive periodontal disease (72, 102).

In the present study *Td* and *Pg* frequency increased without significant differences. This result agrees with previous studies conducted by Guo et al. (123), Naranjo et al. (26), Kim et al. (27), and Thornberg et al. (33). In contrast Ireland et al. (34) published a significant *Td* increase 3 months after appliance installation, followed by a significant decrease to pre-treatment levels 3 months after its removal (34). Lee and collaborators (155) also found a significantly increased *Td* frequency and Naranjo et al. (26) related an increased *Pg* frequency 3 months after therapy begin. According to Liu et al. (122), the subgingival *Pg* amount 6 months after appliance removal was higher than the amount measured before treatment started. This finding may imply a PD potential risk in certain patients.

In relation to *Tf*, another red complex member, in UPJ a significant increase was observed 3 months, and 1 year after therapy begin. In LOJ *Tf* also increased over the observed period, but with a significant increase only at T5. Our results corroborate with those from Naranjo et al. (26), Kim et al. (119) and, Thornberg et al. (33) that also reported a high frequency of patients positive to *Tf* after bracket placement, and during the first 3 months of therapy. Guo et al. (11) also reported higher *Tf* levels after treatment begin but without significant differences. The high *Tf* frequency even 1 year after brackets placement suggests an increased risk to PD development.

5.3.3 Orange complex and bacteria associated to orange complex

The orange complex bacteria *Pi*, *Pm* and *Fs* and the bacteria associated with this complex, *En* and *Cr*, were also assessed in this study. *Cr*, *Fs* and *Pm* were frequently detected at baseline, suggesting that these PP inhabitant the normal subgingival microbiota in adolescents.

Pi increased during the treatment but its frequency returned to similar values to those found at baseline 1 year later. Naranjo et al. (26), Kim et al. (119), Mártha et al. (175) and Sandic et al. (176) reported as well an increased trend of *Pi* during the first 3 months of MBA treatment, but without statistical difference. However, these results are opposite to those found by Ristic et al. (31, 32) and Folco et al. (177) in which *Pi* significant increased 3 months after orthodontic appliance placement.

Pm seems to inhabit the oral commensal flora. In UPJ *Pm* reached its maximum peak 6 weeks after brackets placement followed by a return to pre-treatment levels. In LOJ the maximal peak was 3 months later, returning to initial values 1 year after therapy begin. Both changes occurred in UPJ and LOJ were not significant. None of the studies evaluated in this study had *Pm* levels assessed.

Regarding the frequency of *Fs*, it significantly increased in UPJ after brackets placement until 1 year of therapy and in LOJ increased too but significant just at the last time point. These results agree with those published by Thornberg and coworkers (33) also reported that patient frequency with positive results for *Fs* increases after brackets placement, attaining its maximum values 3 months later. Ristic et al. (31, 32) verified an increase of *Fs* frequency 3 months after therapy start followed by a decrease after 6 months of treatment, which differs from our results, since the frequency of *Fs* remained elevated until the observed period.

Ooshima et al (154) have suggested that *Cr* is a common resident of the normal oral microbiota in heathy children and play a limited role in the PD pathogenesis. However, many other studies report a high prevalence of this specie in gingivitis, periodontitis (44,

84, 178, 179), and in HIV infected patients (86, 87, 180). Ashimoto et al. (178) reported that *Cr* has a positive correlation with the red and orange complexes. In our study the frequency of *Cr* significantly increased in UPJ 6 weeks and 1 year after appliance insertion. In LOJ the differences to baseline were significant 3, 6 weeks, 6 months and, 1 year post bracket placement. In the study from Guo et al. (123) *Cr* showed a temporary increase 1 month subsequently brackets placement, and returned to the pretreatment levels 3 therapy months later. Thornberg et al. (33) report high *Cr* levels 6 months after treatment begin followed by a significantly decreased 3 months after appliance removal. As well as in the study conducted by Kim et al. (27), in which the frequency of *Cr* was elevated before the appliance removal.

The less frequent PP was *En*. In UPJ *En* was detected just in one subject 3 months and by another subject 6 months post therapy begin. In LOJ *En* was isolated in just one subject 6 weeks after appliance placement. Naranjo et al. (26) found a low *En* frequency in their study. The evidence suggests that *En* plays no important role on PP pathogenesis.

Thus, it can be assumed that PP from the orange complex and associate PP are early colonizer that favor red complex bacteria colonization, since red complex PP are rarely found in absence of orange complex members (72).

5.3.4 Green complex

Ec and *Cs* belong to the green complex. According to Ximénez-Fyvie et al. (73), green complex PP are mostly found in supragingival biofilm than in subgingival biofilm. Nevertheless, our findings report a significant increase in the frequency of both *Ec* and *Cs* in subgingival biofilm up to 1 year after therapy begins. Our results concur with those of Thornberg et al. (33) that reported a high frequency of *Ec* until 2 weeks before appliance removal. In opposition, the studies from Kim et al. (119) and Naranjo et al. (26) showed an improvement in *Ec* frequency during the therapy, but without significance.

Some studies have reported a high prevalence of *Ec* in gingivitis, as well as in advanced periodontitis, suggesting that *Ec* can be considered an endogenous pathogen, which occasionally contribute to periodontitis development (178, 179, 181).

Cs is known to inhabit the supragingival plaque and seems to be a colonizer for other complexes bacteria. However, our results showed a significant increase in *Cs* frequency in UPJ after 6 weeks of MBA treatment until the last time point. In LOJ a significant increase is already observed after 3 weeks of appliance insertion and, remained elevated up to 3 months of therapy followed by a small decrease. Mártha et al. found a high *Ec* and *Cs* frequency in subgingival plaque of patients undergoing orthodontic treatment (175).

Based on our microbial analyses, this study results refuse the null hypothesis and confirm a correlation between periodontopathogens before and during treatment.

Since fixed orthodontic appliance treatment can negatively interfere in GI, PI, aMMP-8 concentration and can stimulate growth of certain PPs, preventive measures should be taken before problems occur.

A study published by Bergamo et al. (182) shows a decrease of PP in patients undergoing orthodontic treatment after standardized OHI were given and monitored these patients every 30 days. The authors concluded that PP levels reduction was only viable due to the oral hygiene instructions (OHI) adopted in the study. A study conducted by Marini et al. (183) and another published by Ay et al. (184) also confirmed that OHI and motivation are fundamental in reducing plaque levels of these patients.

Periodontal status in patients submitted to orthodontic treatment requires careful monitoring. Fixed appliances hinder proper buccal hygiene, leading to dental biofilm accumulation, inflammation and bleeding (148, 158). For this reason, appropriate oral hygiene methods and instruments are needed for plaque control (185). Interdental toothbrushes, powered toothbrushes, special types of dental floss, OHI and professional dental cleaning have been proven to be effective on plaque control an orthodontic

patient (143, 186-189). Therefore, OHI, proper hygiene methods, patient motivation and compliance are as important as periodic fixed appliance controls.

6. Conclusion

In summary, it can be concluded that the therapy with the fixed orthodontic appliance may transitionally increase plaque accumulation, aMMP-8 levels, and growth of PP even 1 year after therapy begin. These can result in gingival inflammation but without destruction of periodontal supporting tissue. No significant differences were found between UPJ and LOJ values.

Since changes in clinical parameters and subgingival crevicular fluid increase the risk of periodontal tissue inflammation, proper OHI should be given to orthodontic patients in order to provide a good oral hygiene, constant motivation and continuous plaque control during the entire treatment. Long-term studies are needed to explore the impact of bacterial colonization on periodontal conditions and clinical aspects during the years of orthodontic treatment with fixed appliance and after its removal.

7. Summary

Objectives: The aim of the present prospective study was to investigate changes in clinical parameters, periodontopathogens (PP) levels and active matrix metalloproteinase-8 (aMMP-8) concentration in gingival crevicular fluid of patients before and during treatment with fixed orthodontic appliances.

Material and Methods: Fifty-five adolescents (30 females, 25 males; ages 12-17 years), who were scheduled for fixed orthodontic treatment, were selected and included in this study. Clinical parameters and subgingival samples were obtained at six time points: 1 week before appliance insertion (T0), 3 (T1), 6 (T2) weeks, 3 (T3), 6 (T4) months and 1 year after therapy begin. Gingival index (GI) and plaque index (PI) were assessed to evaluated changes on the clinical status and, subgingival samples were used to analyze changes of aMMP-8 concentration and levels of the following PP: *Agreggatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium spp* (*Fs)*, *Parvimonas micra* (*Pm*) *Campylobacter rectus* (*Cr)*, *Eubacterium nodatum* (*En*), *Eikenella corrodens* (*Ec*) and *Capnocytophaga spp* (*Cs*).

Results: Scores for GI and PI increased after appliance insertion. GI showed a continuous increase from T2 ($p<0.05$) until T5 ($p<0.0001$). PI increased following brackets placement reaching its maximum peak at T3 ($p<0.05$), 3 months after therapy begin. Moreover, a significant increase of aMMP-8 concentration ($p<0.05$) and frequency of *Tf*, *Fs*, *Cr*, *Cs*, and *Ec* was noted. For the other tested bacteria, the frequency tended to increase, but without significant differences. As well, no significant differences were found between upper and lower jaws for all parameters evaluated.

Conclusion: Orthodontic treatment with fixed appliance in adolescents favors dental plaque accumulation and may transitionally increase GI, PI, aMMP-8 concentration and, subsequently, the growth of PP leading to gingival inflammation, even 1 year after therapy began.

7. Zusammenfassung

Ziele: Das Ziel dieser prospektiven Studie war es, Veränderungen der klinischen Parametern, des Niveaus der parodontalen Markerkeime und der aktiven Matrix Metalloproteinase (aMMP-8) Konzentration in der gingivalen Krevikularflüssigkeit von Patienten vor und während der Behandlung mit festsitzenden Apparaturen zu untersuchen**.**

Material und Methoden: Fünfundfünfzig Jugendliche (30 weiblich, 25 männlich; im Alter von 12-17 Jahren), die für eine feste kieferorthopädische Behandlung vorgesehen waren, wurden selektiert und in diese Studie einbezogen. Klinische Parameter und subgingivale Proben wurden zu sechs Zeitpunkten erhoben: Eine Woche vor Einsetzen der festsitzenden Apparatur (T0), 3 (T1), 6 (T2) Wochen, 3 (T3), 6 (T4) Monate und 1 Jahr nach Therapiebeginn. Gingivitis- und Plaque-Indizes wurden auf Veränderungen der klinischen Parameters untersucht und subgingivale Proben wurden entnommen, um Veränderungen der aMMP-8-Konzentration und des Niveaus der folgenden parodontalen Markerkeime zu analysieren: *Agreggatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium spp* (*Fs*), *Parvimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Eubacterium nodatum* (*En*), *Eikenella corrodens* (*Ec*) und *Capnocytophaga spp* (*Cs*) .

Ergebnisse: Die Werte für GI und PI stiegen nach dem Einsetzen der Apparatur. GI zeigte eine kontinuierliche Erhöhung ab T2 ($p<0,05$) bis T5 ($p<0,0001$). PI stieg nach dem Kleben der Brackets an und erreichte seinen Maximum-Peak bei T3 ($p<0,05$), 3 Monate nach Therapiebeginn. Auch eine signifikante Steigerung der aMMP-8-Konzentration und der Frequenz von *Tf*, *Fs*, *Cr*, *Cs* und *Ec* wurde festgestellt. Bei den anderen untersuchten Keimen stieg die Frequenz tendenziell an, aber es wurde kein signifikanter Unterschied festgestellt. Auch wurden keine signifikanten Unterschiede zwischen Ober- und Unterkiefer für alle ausgewerteten Parameter ermittelt.

Fazit: Die kieferorthopädische Behandlung mit festsitzender Apparatur bei Jugendlichen begünstig die Ansammlung von Zahnbelägen und kann die GI-, PI-, aMMP-8-Konzentration und damit das Wachstum von parodontalen Markerkeimen, die zu Zahnfleischentzündungen führen, vorübergehend erhöhen, sogar 1 Jahr nach Therapiebeginn.

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9. Appendix

UNIVERSITÄTSmedizin. MAINZ

Poliklinik für Kieferorthopädie

Studie zur Untersuchung des Mundhygienestatus und der Lebensqualität des Patienten vor, während und nach der festsitzenden kieferorthopädischen Behandlung.

Einwilligungserklärung

für minderjährige Patienten (im Alter von 12-17 Jahren)

Studie zur Untersuchung des Mundhygienestatus und der Lebensqualität des Patienten vor, während und nach der festsitzenden kieferorthopädischen Behandlung.

Voraussetzung für Deine Teilnahme ist, dass Du einverstanden bist. Wenn ja, bitten wir Dich, auf diesem Blatt zu unterschreiben. Du bestätigst uns damit, dass Du an der Studie teilnehmen möchtest und weißt, dass dies freiwillig ist, alle Deine Fragen zu Deiner Zufriedenheit beantwortet wurden und Du genügend Zeit hattest, Deine Teilnahme zu bedenken. Du kannst aber auch später zu jeder Zeit sagen, dass Du nicht mehr an der Studie teilnehmen möchtest. Du wirst deshalb auch keine Nachteile für Deine medizinische Behandlung haben.

Ich erkläre mich freiwillig bereit, an der oben genannten Studie teilzunehmen.

Mit der Erhebung und Verwendung meiner Daten wie in der mir ausgehändigten Informationsschrift beschrieben bin ich einverstanden.

_____
Name des Jugendlichen in Druckbuchstaben

geb. am ..... Teilnehmer-Nr. .....

....
Ort, Datum Unterschrift des **Jugendlichen**

Ich habe das Aufklärungsgespräch geführt und die Einwilligung des Jugendlichen eingeholt. Ich habe mich davon überzeugt, dass der Jugendliche alles verstanden hat, keine weiteren Fragen mehr hat und seiner Teilnahme zustimmt.

_____
Name des Studienarztes in Druckbuchstaben

_____
Unterschrift des aufklärenden **Studienarztes**

Figure 30: Declaration of consent

Klinik und Polikliniken für Zahn-, Mund- und Kieferkrankheiten (ZMK)

UNIVERSITÄTSmedizin. MAINZ

ANAMNESE - ERHEBUNGSBOGEN

Sehr verehrte Patientin, sehr geehrter Patient,

bitte füllen Sie diesen Fragebogen sorgfältig aus. Sollte der Platz für Zusatzangaben nicht ausreichen, benutzen Sie bitte unter Angabe des Punktes die Rückseite des Blattes. Der Fragebogen wird Ihrer Karteikarte beigefügt. Sollten Sie Schwierigkeiten mit der Beantwortung einzelner Fragen haben, helfen wir Ihnen gerne!

Name:_____ **Vorname:**_____ **Geb.-Datum:**_____

1. Haben Sie zu hohen oder zu niedrigen Blutdruck? ( ) nein ( ) ja,_____
2. Haben oder hatten Sie eine Erkrankung des Herzens? ( ) nein ( ) ja,_____
   - angeborene oder erworbene Herzfehler? ( ) nein ( ) ja,_____
   - Endokarditis (Herzinnenhautentzündung)? ( ) nein ( ) ja,_____
   - Herzoperationen, Herzklappenprothese? ( ) nein ( ) ja,_____
   - Haben Sie einen Herzschrittmacher? ( ) nein ( ) ja,_____
3. Haben oder hatten Sie eine der nachstehend aufgeführten Erkrankungen?
   - Diabetes (erhöhter Blutzucker)? ( ) nein ( ) ja,_____
   - Erkrankung des Blutes? (z.B. langes Nachbluten, Blutgerinnungsstörung) ( ) nein ( ) ja,_____
   - Allergien (z.B. Heuschnupfen) ( ) nein ( ) ja,_____
   - Schilddrüsenerkrankung? ( ) nein ( ) ja,_____
   - Asthma / Lungenerkrankungen? ( ) nein ( ) ja,_____
   - Nervenerkrankung (Depression o.ä.)? ( ) nein ( ) ja,_____
   - Anfallsleiden / Epilepsie? ( ) nein ( ) ja,_____
   - Magen-, Darm-, Leber-, Nierenerkrankung? ( ) nein ( ) ja,_____
4. Haben Sie eine Infektionserkrankung z.B. HIV, Hepatitis, Tuberkulose, andere ? ( ) nein ( ) ja,_____
5. Bestehen derzeit sonstige Erkrankungen? ( ) nein ( ) ja
   Wenn ja, welche? _____
6. Welche Medikamente nehmen Sie derzeit ein?_____
   _____
7. Nehmen Sie Medikamente ein, die Blutgerinnung hemmen? ( ) nein ( ) ja,_____
8. Vertragen Sie bestimmte Medikamente nicht? ( ) nein ( ) ja
   Wenn ja, welche? _____
9. Wann wurden Sie zum letzten Mal geröntgt?_____
10. Waren Sie innerhalb der letzten Jahre im Krankenhaus oder in ärztlicher Behandlung? ( ) nein ( ) ja
    Wenn ja, weshalb? _____
11. Für Patientinnen: Sind Sie schwanger? ( ) nein ( ) ja,_____

Mainz, _____ _____
Datum Unterschrift

UKM 19-084 Stand 2/2012

Figure 31: Anamnesis Formulary from the University of Medicine Mainz - Clinic for Dental, Oral and Maxillofacial Diseases.

K

Anamnese

Betr. : Patient.....Alter:.....Jahre

Damit wir die Ursachen der Kieferanomalie bei Ihrem Kind besser erkennen können, bitten wir Sie, die folgenden Fragen möglichst genau zu beantworten.
Kreuzen Sie die zutreffenden Kästchen an, bzw. unterstreichen Sie die entsprechenden Textteile und schreiben Sie die Antwort auf die punktierte Linie.

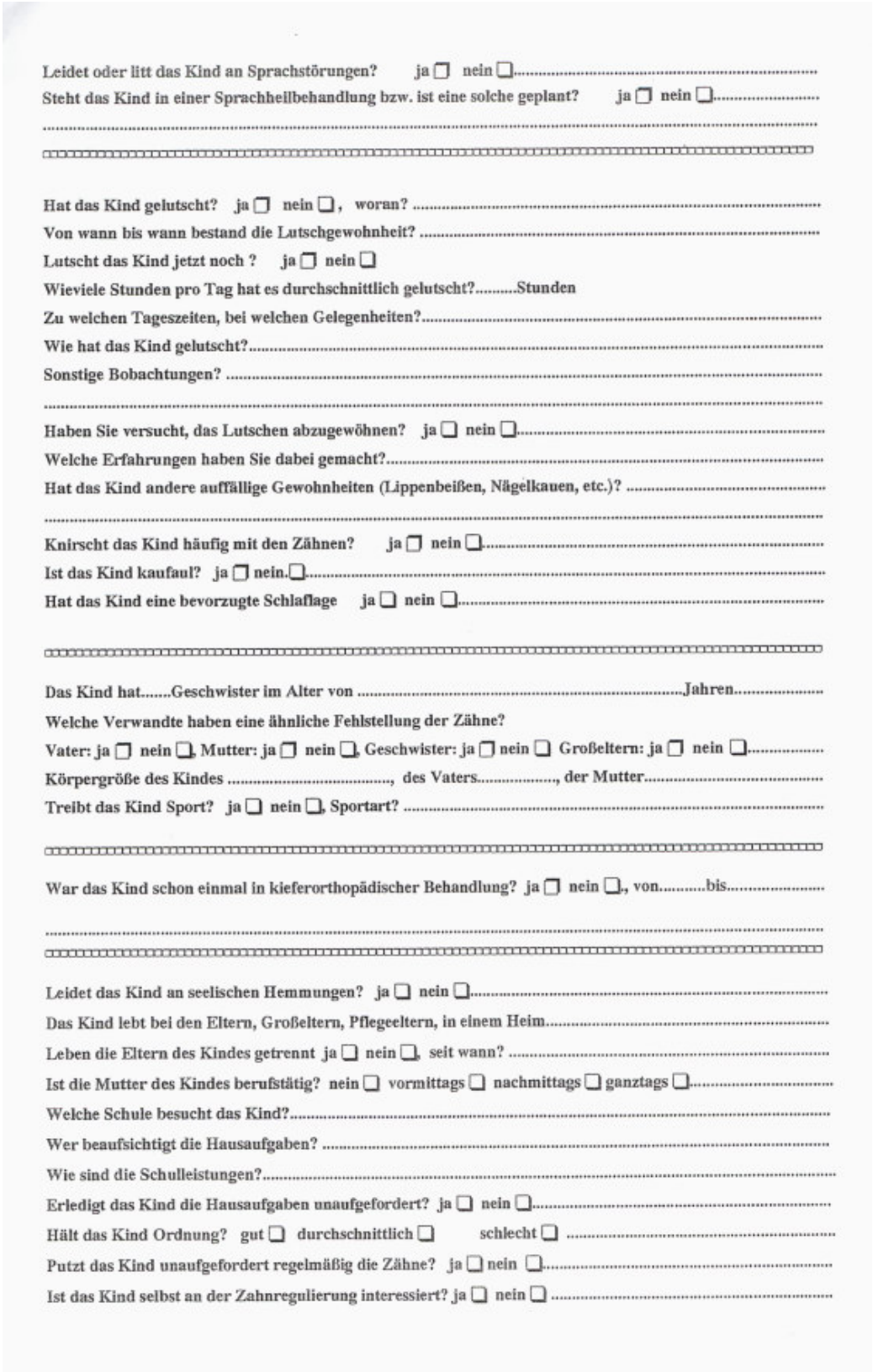
War die Schwangerschaft gestört? ja ☐ nein ☐.....
Verlief die Geburt normal? ja ☐ nein ☐.....
Wievielte Geburt?.....
Wieviele Monate wurde das Kind gestillt? ..... Monate .....
Wann lernte das Kind laufen?.....
In welchem Alter konnte das Kind die ersten Worte sprechen?.....
In welchem Alter erschien der erste Zahn (Milchzahn)?.....
Wie standen die Milchzähne?.....
Wann stellten sich die ersten bleibenden Schneidezähne ein?.....

Hatte das Kind einen Unfall? ja ☐ nein ☐, in welchem Alter?.....
Sind dabei Zähne beschädigt worden? ja ☐ nein ☐.....
Sind Milchzähne längere Zeit vor dem Durchbruch ihrer Nachfolger gezogen worden? ja ☐ nein ☐.....
Sind bereits bleibende Zähne gezogen worden? ja ☐ nein ☐ ....

Welche Krankheiten bzw. Operationen hat das Kind durchgemacht? .....
.....
Leidet das Kind derzeit an einer ernsteren oder länger dauernden Erkrankung? ja ☐ nein ☐.....
Hat das Kind zur Zeit eine möglicherweise ansteckende Krankheit? ja ☐ nein ☐.....
Leidet das Kind unter einer Allergie? ja ☐ nein ☐ .....

Hält das Kind den Mund meist offen? ja ☐ nein ☐ .....
Schläft es mit geöffnetem Mund? ja ☐ nein ☐, schnarcht es? ja ☐ nein ☐.....
Ist das Kind häufig erkältet? ja ☐ nein ☐ .....
War das Kind schon beim Halsen-Nasen-Ohrenarzt ? ja ☐ nein ☐ .....
Wann?.....Was wurde gemacht? .....

Figure 32: Anamnesis Formulary from the Department of Orthodontics of the Johannes Gutenberg University of Mainz.



Leidet oder litt das Kind an Sprachstörungen? ja ☐ nein ☐.....

Steht das Kind in einer Sprachheilbehandlung bzw. ist eine solche geplant? ja ☐ nein ☐.....

.....

Hat das Kind gelutscht? ja ☐ nein ☐, woran? .....

Von wann bis wann bestand die Lutschgewohnheit? .....

Lutscht das Kind jetzt noch ? ja ☐ nein ☐

Wieviele Stunden pro Tag hat es durchschnittlich gelutscht?.....Stunden

Zu welchen Tageszeiten, bei welchen Gelegenheiten?.....

Wie hat das Kind gelutscht?.....

Sonstige Bobachtungen? .....

.....

Haben Sie versucht, das Lutschen abzugewöhnen? ja ☐ nein ☐.....

Welche Erfahrungen haben Sie dabei gemacht?.....

Hat das Kind andere auffällige Gewohnheiten (Lippenbeißen, Nägelkauen, etc.)? .....

.....

Knirscht das Kind häufig mit den Zähnen? ja ☐ nein ☐.....

Ist das Kind kaufaul? ja ☐ nein.☐.....

Hat das Kind eine bevorzugte Schlaflage ja ☐ nein ☐.....

Das Kind hat.....Geschwister im Alter von .....Jahren.....

Welche Verwandte haben eine ähnliche Fehlstellung der Zähne?

Vater: ja ☐ nein ☐, Mutter: ja ☐ nein ☐, Geschwister: ja ☐ nein ☐ Großeltern: ja ☐ nein ☐.....

Körpergröße des Kindes ....., des Vaters....., der Mutter.....

Treibt das Kind Sport? ja ☐ nein ☐, Sportart? .....

War das Kind schon einmal in kieferorthopädischer Behandlung? ja ☐ nein ☐., von.....bis.....

.....

Leidet das Kind an seelischen Hemmungen? ja ☐ nein ☐.....

Das Kind lebt bei den Eltern, Großeltern, Pflegeeltern, in einem Heim.....

Leben die Eltern des Kindes getrennt ja ☐ nein ☐, seit wann? .....

Ist die Mutter des Kindes berufstätig? nein ☐ vormittags ☐ nachmittags ☐ ganztags ☐.....

Welche Schule besucht das Kind?.....

Wer beaufsichtigt die Hausaufgaben? .....

Wie sind die Schulleistungen?.....

Erledigt das Kind die Hausaufgaben unaufgefordert? ja ☐ nein ☐.....

Hält das Kind Ordnung? gut ☐ durchschnittlich ☐ schlecht ☐ .....

Putzt das Kind unaufgefordert regelmäßig die Zähne? ja ☐ nein ☐.....

Ist das Kind selbst an der Zahnregulierung interessiert? ja ☐ nein ☐ .....

Figure 33: Anamnesis Formulary from the Department of Orthodontics of the Johannes Gutenberg University of Mainz.

Figure 34: Flyer with oral hygiene and cleaning instructions.