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PERIODONTAL AND MICROBIAL IMPLICATIONS OF ORTHODONTIC TREATMENT WITH FIXED APPLIANCES

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TABLE OF CONTENTS

DANKWOORD **1**
TABLE OF CONTENTS **3**
LIST OF ABBREVIATIONS **7**
SAMENVATTING **9**
SUMMARY **13**
CHAPTER I: GENERAL INTRODUCTION **17**
I.1 Background 18
I.2 Pathogenesis of gingivitis 19
I.2.a Dental plaque 19
I.2.b Host reaction 19
I.3 Orthodontic treatment 20
1.3.a Short term effects 20
I.3.a.1 Microbiology 20
I.3.a.2 Clinical periodontal parameters 22
I.3.a.3 Histology 24
I.3.b Long term effects 25
I.3.c Different orthodontic materials 25
I.4 Conclusions 27
I.5 Aims 28

CHAPTER II: INFLUENCE OF BRACKET DEGIGN ON MICROBIAL AND PERIODONTAL PARAMETERS *IN VIVO* 29

II.1 Introduction 31

II.2 Material and methods 32

II.2.a Subjects 32

II.2.b Experimental design 33

II.2.b.1 Bracket placement/removal 35

II.2.b.2 Microbiology 36

II.2.b.3 Crevicular fluid 36

II.2.b.4 Periodontology 37

II.2.b.5 Culture techniques 37

II.2.b.6 Scanning electron microscopy 38

II.3 Statistics 38

II.4 Results 39

II.4.a Microbiology 39

II.4.b Crevicular fluid 41

II.4.c Periodontology 42

II.4.d Scanning electron microscopy 43

II.5 Discussion 44

II.6 Conclusion 49

CHAPTER III: MICROBIAL ADHESION ON DIFFERENT BRACKET TYPES *IN VITRO* 51

III.1 Introduction 53

III.2 Material and methods 53

III.2.a Brackets 53

III.2.b Medium 55

III.2.c Bracket removal 55

III.2.d Culture techniques 55

III.3 Statistics 56

III.4 Results 56

III.4.a Microbiology 56

III.4.a.1 CFU aerobe 56

III.4.a.2 CFU anaerobe 56

III.4.a.3 CFU ratio 57

III.4.a.4 CFU *P. Intermedia* 58

III.5 Discussion 60

III.6 Conclusion 63

CHAPTER IV: SHORT TERM MICROBIAL AND PERIODONTAL CHANGES DURING TREATMENT WITH FIXED ORTHODONTIC APPLIANCES 65

IV.1 Introduction 67

IV.2 Material and methods 67

IV.2.a Subjects 67

IV.2.b Experimental design 68

IV.2.b.1 Band placement 70

IV.2.b.2 Bracket placement 70

IV.2.b.3 Microbiology 71

IV.2.b.4 Crevicular fluid 71

IV.2.b.5 Periodontology 71

IV.2.b.6 Culture techniques 71

IV.3 Statistics 71

IV.4 Results 72

IV.4.a Microbiology 72

IV.4.b Crevicular fluid 72

IV.4.c.Periodontology 74

IV.5 Discussion 74

IV.6.Conclusion 80

CHAPTER V: CHANGES IN GCF FLOW AND COMPOSITION AFTER ONE YEAR OF ORTHODONTIC TREATMENT, A PREDICTIVE VALUE? 81

V.1 Introduction 83

V.2 Material and methods 83

V.2.a Subjects 83

V.2.b Experimental design 84

V.2.b.1 Band placement 85

V.2.b.2 Bracket placement 85

V.2.b.3 Microbiology 85

V.2.b.4 Crevicular fluid 85

V.2.b.5 Periodontology 86

V.2.b.6 Culture techniques 87

V.3 Statistics 87

V.4 Results 88

V.4.a Microbiology 88

V.4.b Crevicular fluid 88

V.4.c.Periodontology 90

V.5 Discussion 91

V.6.Conclusion 95

CHAPTER VI: LONGITUDINAL CHANGES IN MICROBIOLOGY AND CLINICAL PERIODONTAL PARAMETERS AFTER REMOVAL OF FIXED ORTHODONTIC APPLIANCES 97

VI.1 Introduction 99

VI.2 Material and methods 100

VI.2.a Subjects 100

VI.2.b Experimental design 100

VI.2.b.1 Band placement 101

VI.2.b.2 Bracket placement 101

VI.2.b.3 Debonding 101

VI.2.b.4 Three months after debonding 102

VI.2.b.5.Microbiology 102

VI.2.b.6 Crevicular fluid 102

VI.2.b.7 Periodontology 102

VI.2.b.8 Culture techniques 102

VI.3 Statistics 102

VI.4 Results 103

VI.4.a Microbiology 103

VI.4.b Crevicular fluid 104

VI.4.c.Periodontology 104

VI.5 Discussion 107

VI.6.Conclusion 109

CHAPTER VII: GENERAL DISCUSSION 111

VII.1 Introduction 112

VII.2 Influence of bracket design 113

VII.3 Microbial and periodontal changes during orthodontic treatment 116

VII.4 Immunological reaction in the GCF during orthodontic treatment 119

VII.5 Microbial and periodontal changes after debonding 121

REFERENCES 125

CURRICULUM VITAE 137

LIST OF ABBREVIATIONS

BHI	Brain heart infusion
bop	Bleeding on probing
CFU	Colony forming units
CFU ratio	Aerobe CFU/anaerobe CFU
DPCM	Differential phase contrast microscopy
ELISA	Enzyme-linked immunosorbent assay
GCF	Gingival crevicular fluid
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
IFN	Interferon
IL	Interleukin
IP	Interferon-inducible Protein
kDA	Kilo Dalton
m	Mean
MCP	Monocyte Chemotactic Protein
PBS	Phosphate buffered saline
PDL	Periodontal ligament
PMN	Polymorphonuclear cell
ppd	Periodontal probing depth
rpm	Revolutions per minute
RTF	Reduced transport fluid
SD	Standard deviation
SEM	Scanning electron microscopy
TDI	Tissue destruction index
TNF	Tumor necrosis factor

SAMENVATTING

Dit onderzoeksproject is opgestart om de mogelijke gevolgen van orthodontisch behandelen met vaste apparatuur uit te klaren. Meer specifiek worden de microbiële, parodontologische en immunologische veranderingen geëvalueerd. De effecten van verschillende brackettypes (verschillende blokjes) worden onder de loep genomen, zowel *in vivo* als *in vitro*. Verder worden de korte termijn (tijdens de behandeling) en de langere termijn (3 maanden na de behandeling) gevolgen van een orthodontische behandeling prospectief opgevolgd.

Hoofdstuk I belicht de achtergrond van het onderzoek en evalueert het niveau van de bewijskracht van recente orthodontische en parodontologische literatuur betreffende dit onderwerp.

Hoofdstuk II presenteert een geblindeerd, gerandomiseerd *de novo* plaquegroei experiment met “split-mouth” ontwerp. Gedurende een periode van zeven dagen zonder poetsen worden de microbiële en parodontologische veranderingen rond tanden met twee verschillende brackettypes met elkaar en met controletanden vergeleken. Er blijken significante verschillen wat betreft de microbiële samenstelling van de tandplaque te zijn tussen de verschillende brackettypes onderling alsook tussen de gebondeerde en controle- elementen. Significant hogere tellingen voor de aerobe en anaerobe bacteriën zijn zichtbaar voor de met Speed® gebondeerde tanden vergeleken met de GAC® gebondeerde en controle tanden. De Speed® tanden vertoonden ook een lagere CFU ratio aeroob/anaeroob dan de GAC® en controle tanden, hetgeen indicatief is voor een meer pathogeen milieu rond de Speed® gebondeerde tanden. Deze microbiële verschillen worden gevolgd door een gelijkaardige trend in

creviculaire vloeistof stroom, pocketdiepte en neiging tot bloeden na sonderen. Dit geeft aan dat de variatie in bracketontwerp duidelijke repercussies heeft op de klinische reactie van de actieve orthodontische patiënt. Verder onderzoek in deze richting is zeker noodzakelijk om zo een bracket systeem te kunnen ontwerpen dat weinig tot geen verhoogde plaque opstapeling vertoont.

Hoofdstuk III beschrijft een *in vitro* studie met een duurtijd van 72 uur waarin 7 verschillende brackettypes getest worden. Uit deze studie kunnen we besluiten dat er significante verschillen zijn betreffende de microbiële adhesie tussen de verschillende brackets. De keramische brackets bevinden zich beiden in de hoge adhesie groep, terwijl de conventionele brackets de minste adhesie vertonen. De zelfligerende brackets, die wegens de afwezigheid van een elastomerische ring minder gevoelig zouden zijn voor plaqueadhesie, bevinden zich in de hoge en gemiddelde adhesie groep. Dit betekent dat de elastomerische ring niet de eigenschappen van de bracket overschaduwt. De brackets met de hoogste adhesie vertonen ook de laagste CFU ratio aeroob/anaeroob en dus de meest pathogene microbiële samenstelling van de plaque. De resultaten van deze *in vitro* studie kunnen niet zomaar geëxtrapoleerd worden naar een *in vivo* omgeving, meer klinische studies in dit gebied zijn dus zeker aangewezen.

Hoofdstuk IV presenteert een prospectieve longitudinale studie met een “blind split-mouth” ontwerp waarin gebondeerde (tanden voorzien van een blokje), gebandeerde (tanden voorzien van een bandje) en controletanden met elkaar worden vergeleken. Uit de data verkregen met deze studie kunnen we concluderen dat de plaatsing van beide types orthodontische materialen een negatieve invloed heeft op de microbiële en parodontologische parameters. Verrassend is dat het plaatsen van de banden geen invloed heeft op alle microbiologische en de meeste parodontologische parameters (enkel de pocketdiepte nam toe). Na het plaatsen van de orthodontische draad, zijn er echter wel duidelijk verslechteringen zichtbaar. Dit geeft aan dat het aanbrengen van de draad het reinigen bemoeilijkt en een bijkomende uitlokkende factor is in de ontwikkeling van gingivitis. Gedurende de actieve behandeling vertonen de gebondeerde tanden een betere microbiële en ongeveer dezelfde parodontale status als de gebandeerde tanden. De

controletanden vertonen geen veranderingen in de tijd, waaruit we mogen concluderen dat we te maken hebben met strikt lokale veranderingen.

Enkel de aanwezigheid van een pathogene microbiologie betekent nog niet dat de patiënt parodontitis gaat ontwikkelen. Uit de literatuur blijkt dat dit afhankelijk is van een zeer complexe bacterie gastheer interactie, maar verlies van aanhechting is uiteindelijk niet uit te sluiten.

In **Hoofdstuk V** worden de microbiële en parodontologische veranderingen na een periode van één jaar orthodontische behandeling met vaste apparatuur bekeken en gerelateerd aan de wijzigingen in cytokine concentratie in de creviculaire vloeistof. De IL-6 en IL-8 concentraties voor de behandeling zijn significant predictieve factoren voor inflammatoire parameters tijdens de behandeling. Verhoogde waarden van IL-6 voor de start zijn voorspellend voor verhoogde creviculaire vloeistofstromen na één jaar orthodontische behandeling. Hoge waarden voor de concentratie aan IL-8 op baseline zijn voorspellend voor een verhoogde pocketdiepte na één jaar.

Deze informatie is nieuw en kan bijdragen aan een weloverwogen beslissing om al dan niet te starten met een vaste apparatuurbehandeling, vooral bij deze patiënten die een behandeling overwegen wegens milde esthetische afwijkingen.

Hoofdstuk VI geeft een studie waarin de microbiële en parodontale veranderingen opgevolgd worden gedurende een orthodontische behandeling tot en met drie maanden na behandeling. Dit om te kijken of en in welke mate, de veranderingen die zich tijdens de behandeling voordoen normaliseren na het verwijderen van de orthodontische apparatuur. Uit de gegevens blijkt dat de plaatsing van vaste orthodontische apparatuur een significante impact heeft op zowel de microbiologie alsook op de parodontologische parameters. Alle waarden verslechteren tijdens de behandeling. De supragingivale CFU ratio (aeroob/anaeroob) normaliseert zich 3 maanden na de behandeling. De subgingivale CFU ratio (aeroob/anaeroob) daarentegen blijft significant verhoogd 3 maanden na de behandeling ten opzichte van vóór de behandeling. Dit verschil is mogelijk te verklaren door het feit dat de supragingivale flora meer onder invloed staat van het poetsgedrag. De gebondeerde en gebandeerde tanden onderling, vertonen geen significante microbiële verschillen, niet gedurende de orthodontische behandeling en ook niet 3

maanden na de behandeling. De parodontale parameters neigen tot normalisatie na het verwijderen van de orthodontische apparatuur, maar de meeste parodontale waarden blijven significant hoger dan de waarden vóór behandeling. Om definitieve veranderingen uit te sluiten zou het opvolgen van deze groep over een grotere periode zeer interessant zijn.

In **Hoofdstuk VII** wordt een algemene beschouwing gegeven van het onderzoeksproject en wordt het gekaderd in de heden ten dagen beschikbare literatuur.

SUMMARY

This doctorate project is performed to investigate the possible effect of orthodontic treatment with fixed orthodontic appliances on the microbial composition of the dental plaque, clinical periodontal parameters and the GCF immunological response. The effects of different designs of orthodontic brackets are evaluated *in vitro* and *in vivo*. The short term (during treatment) and long term (3 months after treatment) effects are studied.

Chapter I elucidates the background of the study and gives an evaluation of the level of evidence of current orthodontic/periodontal literature.

In **Chapter II**, a randomized examiner blinded *de novo* plaque growth experiment with split-mouth design is employed to study the effects of different bracket designs on microbial and periodontal parameters during a period of seven days without brushing. Significant differences are observed for the microbial adhesion between the bonded and non bonded control teeth and also between the teeth bonded with Speed® and GAC® brackets. Significantly higher aerobe as well as anaerobe CFU counts are present on teeth bonded with Speed® compared to GAC® and controls. The Speed® bonded teeth also show lower CFU ratio aerobe/anaerobe than GAC® bonded and control teeth. These differences are accompanied by similar trends in GCF flow and probing depth. This indicates that the variation in bracket design has repercussions on the clinical response of the patient to orthodontic treatment. Further research should focus on the potentially different microbial/periodontal complications of different orthodontic bracket systems used in treatment with fixed appliances, in such a way that brackets can be designed to reduce plaque adhesion.

Chapter III is the description of a short term *in vitro* study testing different brackets. From this study we can conclude that orthodontic brackets serve as

different loci for biofilm formation. The ceramic brackets are both in the high adhesion group while the conventional twin brackets do present the least microbial adhesion. The self-ligating brackets were in the high and intermediate adhesion group, supporting the idea that the elastomeric ligature does not overshadow the impact of the bracket base. The brackets showing the higher CFU counts did also present the lower CFU ratio aerobe/anaerobe, indicating more pathological environments.

As the results from an *in vitro* study can not be directly extrapolated to an *in vivo* setting, further comparisons by means of a RCT with several different bracket types could fill the lacuna in this field of clinical orthodontics.

Chapter IV presents a prospective longitudinal study with an examiner blinded split-mouth design in which the banded and bonded teeth were compared to control teeth. From the data obtained with this study we can conclude that the placement of both types of orthodontic attachments has a negative influence on both the microbial and the clinical periodontal parameters. Surprisingly, the placement of orthodontic bands alone does not have significant effects on the microbiota and most clinical parameters (only the probing depths increased significantly), but after insertion of the orthodontic wire, changes do occur and the wire might be the trigger in the development of gingival inflammation. During treatment, the banded sites show a better microbial status and approximately the same clinical periodontal status compared to the bonded sites. Ultimately, the control sites do not show any significant changes over time, from which we may conclude that the alterations occurring around the bracket/band bearing teeth are strictly local.

The mere presence of pathogenic microbiota does not mean that the patient will develop periodontal disease, since the latter depends on a complex bacteria-host interaction that modulates the host's individual response leading to inflammation and possibly, loss of attachment.

In **Chapter V,** the microbiological and clinical periodontal changes after placement of orthodontic attachments over a 1 year period are monitored and linked to alterations in cytokine concentrations in the GCF. The results clearly show that IL-6 and IL-8 concentrations before orthodontic treatment are significant predictive factors for some inflammatory parameters during treatment. Increased values of IL-6 at baseline were correlated with increased

GCF flows after one year of orthodontic therapy. High levels of IL-8 at baseline are predictive for higher probing depths after one year treatment. This information is new and could contribute to the decision whether or not to start an orthodontic treatment in susceptible patients. More research in this area is needed in order to elucidate the predictive value of those biomarkers. They might give clinical orthodontist the possibility to screen the patients before active treatment in order to decide whether or not it is indicated to start orthodontic treatment, especially in patients who consider treatment for mild aesthetic deviations.

Chapter VI presents a study in which the microbial an clinical periodontal changes after the placement of orthodontic bands and brackets are monitored and to check whether or not these parameters normalize after termination of the orthodontic therapy. The placement of fixed orthodontic appliances has a significant impact on both the microbial and clinical periodontal parameters. All values worsened significantly between placement of the bands and brackets and termination of the orthodontic treatment. The supragingival CFU ratio (aerobe/anaerobe) normalizes after 3 months posttreatment, possibly because these sites are more sensitive to changes in oral hygiene. The subgingival CFU (aerobe/anaerobe) ratio on the other hand remains significantly lower at 3 months posttreatment compared to pretreatment. Indicating that the changes induced by orthodontic treatment are partially irreversible (over this 3 month period). The bonded and the banded sites did not present different microbial changes, neither during the orthodontic treatment nor during the 3 months posttreatment period. The periodontal values tend to normalize after debonding, but most periodontal values remain significantly elevated at 3 months posttreatment compared to pretreatment. These measurements should be repeated over a longer period of time to elucidate the long term changes.

In **chapter VII** the results of the PhD project are discussed and compared to the recent literature available.

CHAPTER 1:

GENERAL INTRODUCTION

GENERAL INTRODUCTION

I.1 Background

The past decades have witnessed a steady increase of patients undergoing orthodontic treatment with fixed appliances (Shaw et al., 1991). Most of these patients are treated to improve dentofacial aesthetics. Only a minority of them actually required treatment from a medical or dental point of view. In many cases they are purely aesthetic (van Gastel et al., 2007a). Periodontal disadvantages of orthodontic treatment with fixed appliances need to be characterized because the damage may be considerable and the benefits from orthodontic treatment questionable.

Misalignment of the dentition is one of the possible dental justifications for orthodontic treatment, nevertheless there is a controversy about the impact of crowding and malocclusion on the accumulation and maturation of dental plaque (Socransky, 1970; Socransky, 1977).

Starting an orthodontic treatment for purely aesthetic reasons can only be supported when the treatment itself does not harm the (periodontium of the) patient. The aetiology and pathogenesis of periodontal diseases are known to be multifactorial, but dental plaque certainly is an essential precursor. Hence any factor presumed to promote plaque accumulation or to make the removal more difficult might contribute to a local increased risk for periodontal disease also. The orthodontic brackets and bands are known to be one of those plaque accumulation enhancing factors. Despite the widespread use of fixed appliances to solve orthodontic problems, there is only little scientific evidence available on the periodontal implications of bracket placement and the long-term effects of an orthodontic treatment.

I.2 Pathogenesis of gingivitis

I.2.a Dental plaque

Dental plaque is a highly complex biofilm that provides nutrients and protection for periodontopathogenic bacteria (Marsh, 2005). It is the primary cause of gingivitis and the possible transition to periodontitis (Löe, 1965). The Gram-positive and mostly aerobic micro-organisms that initially colonize intra-oral hard surfaces are replaced by predominantly Gram-negative and anaerobic micro-organisms (Socransky et al., 1998; Socransky & Haffajee, 2005). *Tannerella forsythia, Porphyromonas gingivalis, Actinobacillus Actinomycetemcomitans* and *Prevotella intermedia* are found more frequently in patients with gingivitis and periodontitis than in healthy subjects (Darveau et al., 1997).

Thus both the quantity as well as the quality of plaque, are important factors in the onset of periodontal disease and are influenced by many factors including surface characteristics (Quirynen et al., 1988; Quirynen et al., 1989; Quirynen et al., 1990). Especially surface roughness and surface free energy were found to be positively correlated with the plaque growth rate (Quirynen & Bollen, 1995) and the presence of gingival inflammation will further increase plaque growth (Quirynen et al., 1991; Ramberg et al., 1995).

I.2.b Host reaction

In 1976 Loesche postulated his 'specific plaque hypothesis' and stated that certain micro-organisms in the dental plaque are pathogenic and that overgrowth of these species would result in increased signs of gingival inflammation (Loesche, 1976). The increase in gingival inflammation is due to the host's immune response. The transition from a reversible gingivitis to an irreversible periodontitis is probably regulated by the bacteria-host reaction. For example, Fransson et al. (1996,1999) examined the host reaction to dental plaque in gingival specimens taken from young and old individuals, and reported that the inflammatory lesions were more marked and had higher B-cell densities in the older subjects. They postulated that these differences were due to the different experiences of the young and old to microbial challenges (Fransson et al., 1996; Fransson et al., 1999).

Family studies suggest that environmental factors (e.g. oral hygiene and smoking) are major determinants of variance in adult periodontitis (Haffajee et al., 1991). Data from twin studies indicate that about half the population variance in periodontitis can be attributed to genetic factors (Corey et al., 1993; Michalowicz et al., 1991; Michalowicz, 1993; Michalowicz, 1994a; Michalowicz, 1994b; Michalowicz et al., 1999; Michalowicz et al., 2000). Moreover, there is mounting evidence that genetic variations in or near the cytokine genes may influence the inflammatory response in individuals with periodontitis (Cullinan et al., 2001; Lopez et al., 2005; Taylor et al., 2004). The accumulation of dental plaque is not the only parameter responsible for the initiation of gingivitis and for the possible transition to periodontitis. This makes it difficult to find direct interactions (or associations) between orthodontic anomalies, such as crowding, and periodontal conditions.

I.3 Orthodontic treatment

I.3.a Short-term effects

I.3.a.1 Microbiology

The placement of orthodontic appliances is known to create a favourable environment for the accumulation of microbiota and food residues which, in time, may cause caries or exacerbate any preexisting periodontal disease. The microbiological changes after bracket placement became a topic of interest during the late 1980's. Initially cariogenic species such as *Streptococcus mutans* and *Lactobacillus* species and the subsequent decalcification of enamel were the main fields of interest among investigators (Forsberg et al., 1991; Rosenbloom & Tinanoff, 1991). Later on, the more complex system of periodontopathogenic microbes and the changes after bracket placement became the main topics of interest (Paolantonio et al., 1996; Paolantonio et al., 1999; Petti et al., 1997).

Naranjo et al. (2006) observed a transition in subgingival dental plaque after the placement of brackets, the plaque index and gingivitis index increased significantly. *P. gingivalis, P. intermedia, P. nigrescens, T. forsythia,* and

Fusobacterium species were significantly elevated in the experimental group after bracket placement compared with the control group without orthodontic therapy. Superinfecting microorganisms such as *Enterobacter cloacae, Klebsiella oxytoca, Klebsiella pneumoniae*, and *Serratia marcescens* were also found (Naranjo et al., 2006). Lee et al. (2005) succeeded in detecting significant differences in the subgingival dental plaque retrieved from gingivitis lesions in patients with and without orthodontic fixed appliances. *T. forsythia, T. denticola, and P. nigrescens* were significantly more common in the samples obtained from the orthodontic patients than in the samples obtained from the non-orthodontic control patients (Lee et al., 2005). These results indicate that the local changes associated with orthodontic brackets affect the prevalence of periodontal pathogens in dental plaque and induces signs of gingivitis. However, no differences in periodontal pocket depth were witnessed. This microbial change is specifically restricted to the dental plaque from orthodontic appliance bearing teeth. The presence of orthodontic bands and brackets therefore cannot affect the microbiologic condition of the whole mouth (Lee et al., 2005; Naranjo et al., 2006).

Huser et al. (1990) performed clinical and bacterial examinations before the beginning of treatment and after placement of the orthodontic appliances (full banded arches) up to 90 days. They again showed significantly increased plaque indices and bleeding scores on banded teeth compared with control sites, the probing depth however remained within normal values for both test and control groups. The composition of dental plaque was only determined by dark-field microscopy and showed significant shifts in the test sites after banding, with an increase in the percentage of spirochetes, motile rods, filaments, and fusiforms. During the same period no significant changes in the bacterial distribution were observed in the control group (Huser et al., 1990). Amanti-Kipioti et al. (1987) compared six banded patients to controls and saw an increase in black pigmented bacteria without an increase in gingivitis and plaque indices, the pocket depth however did increase significantly (Amanti-Kipioti et al., 1987). Petti et al. (1997) performed a similar study with a duration of 6 months. They compared the influence of fixed appliances with removable orthodontic appliances on supra and subgingival microbiota. Their data suggest that in well motivated patients with good oral hygiene, gingivitis and periodontitis

did not occur during the first six months of treatment. The significant modification of oral microbiota, shown by subjects with fixed appliances however, suggests that the risk for gingivitis in the following months of therapy is still high and the risk for periodontitis cannot be excluded (Petti et al., 1997).

I.3.a.2 Clinical periodontal parameters

The placement of orthodontic attachments such as bands and brackets creates new retentive locations, increases plaque accumulation and thereby promotes the inflammatory response (Alexander, 1991). Earlier procedures such as full banding is known to give a generalized form of gingivitis within a short period of time, even with good oral hygiene (Figure I.1) (Kloehn & Pfeifer, 1974; Zachrisson & Zachrisson, 1972). This reaction is characterized clinically by increased signs of inflammation, gingival swelling and pseudo pocket formation, particularly at the proximal areas. The reductions in pocket depths following termination of active treatment are not accompanied by concomitant reductions in the distances to a stable reference point. This indicates that the increased probing depths during treatment are mainly due to oedematous swelling and tissue accumulation during tooth movement, and not to an apical movement of the gingival pocket epithelium. Kouraki et al. followed thirty adolescent patients, who underwent orthodontic treatment for an average period of 27.5 months, and found gingival enlargements in all patients after treatment completion (Kouraki et al., 2005). The average percentage of papillae affected in a patient was 46% at bracket removal compared to 29% pretreatment. After removal of the brackets a significant reduction in gingival enlargements was shown, but the values two to three months posttreatment remained significantly higher than pretreatment (Kouraki et al., 2005). Ramadan measured concentrations of chromium and nickel in saliva after the placement of fixed orthodontic appliances and their effects on gingival tissues during orthodontic treatment (Ramadan, 2004). Three months after treatment initiation, 20% of females and 10% of males in his study showed gingival swelling which was explained as an allergic reaction. This gingival enlargement disappeared only partly one month after appliance removal. These studies are in disagreement however, with other studies in which the gingival enlargement observed during orthodontic treatment (Figure 1) was completely reversible upon removal of the orthodontic

appliances for all patients examined (Alexander, 1991; Amanti-Kipioti et al., 1987; Zachrisson & Zachrisson, 1972). Although most authors agree that the changes during orthodontic treatment are completely reversible, some studies do report of attachment loss during orthodontic treatment with fixed appliances (Zachrisson & Alnaes, 1973; Zachrisson & Alnaes, 1974; Zachrisson, 1976).



Figure I.1: Clinical example of a patient with a localized hypertrophic reaction of the gingiva immediately after removal of the fixed orthodontic appliances. The increased probing depths are due to pseudo-pocket formation.

The degree of gingival swelling can be used as an indicator of gingival inflammation. A reliable and earlier indicator of inflammation is the crevicular fluid flow (µl/min) and the cytokines present in this fluid. Cytokines are low-molecular weight proteins (mw < 25 kDa) produced by cells that regulate or modify the action of other cells in an autocrine (acting on the cell of origin) or paracrine (acting on adjacent cells) manner. The definition includes the interleukins (ILs), tumor necrosis factors (TNFs), interferons (IF), growth factors (GF), and colony stimulating factors (CSF). Cytokines present in the crevicular fluid, have been known to mediate the hosts immunological responses to exogenous antigens (Griffiths, 2003). Recently, it was found that they are also

produced by connective tissue cells such as fibroblasts and osteoblasts and are involved in normal physiological turnover and bone remodelling (Ren & Vissink, 2008). A major difficulty in understanding cytokine biology is the sheer number and complexity of these factors. Another problem is that several factors such as IL-1 and TNF exhibit overlapping biological activities (redundancy) and many have multiple biological effects (pleiotropy). This makes it difficult to make a distinction between the cytokine response to inflammation and to tooth movement during orthodontic treatment in clinical studies.

I.3.a.3 Histology

Zachrisson demonstrated in a longitudinal study of gingival biopsies from buccal sites of molars that shortly after the insertion of bands the gingival connective tissue was infiltrated by inflammatory cells (Zachrisson & Zachrisson, 1972). The dominant cell type in the first month was of the lymphocyte line, but there was a subsequent shift to plasma cells. The leukocytic infiltrate also differed regarding the topography of the different cell types: while plasma cells were located more centrally in the connective tissue, lymphocytes and PMNs were located more superficially, close to the epithelium. After band removal the inflammatory gingival lesions gradually decreased, with acanthosis of the sulcus epithelium persisting sporadically (Zachrisson & Zachrisson, 1972). Diedrich et al. showed the histopathological image of an established gingival lesion: detached and severely damaged epithelial attachment, subgingival plaque, massive leukocyte infiltration (mainly lymphocytes, PMNs and plasma cells) and loss of collagen resulting in destruction of the intrapapillary fibre apparatus. The transseptal fibres, the last protective barrier against microbial aggression towards the alveolar margin, were permeated to varying degrees by cellular infiltrates especially at the interdental spaces due to the subgingival position of the band margins. At one of these sites, the connective tissue attachment was severely damaged resulting in apical migration of the pocket epithelium leading to a transition from chronic gingivitis to an initial irreversible periodontal lesion (Diedrich et al., 2001).

I.3.b Long term effects

In order to elucidate the long term effects of orthodontic treatment, Sadowsky et al. conducted a retrospective study in which they compared 96 subjects who had received orthodontic treatment during adolescence with a control group consisting of 103 subjects (matched for race, sex, age, socio-economic status, oral hygiene and dental awareness) but with malocclusions that had not been orthodontically treated (Sadowsky & BeGole, 1981). In general, they did not detect significant differences between the two groups concerning the general prevalence of periodontal disease (conform the tissue destruction index (T.D.I.)), but a more detailed analysis showed that the subjects in the orthodontically treated group showed slightly elevated T.D.I. scores in the maxillary posterior and mandibular anterior teeth as compared to the control group. The results suggest that orthodontic treatment in adolescence is not a major factor in determining the long-term periodontal health status. Neither does the lack of orthodontic treatment in adolescence appear to influence subsequent development or non-development of periodontal disease in adult life (Sadowsky & BeGole, 1981). Polson et al. (1988) drew the same conclusions after performing a comparable study in one hundred and twelve orthodontically treated and one hundred and eleven control subjects (Polson et al., 1988). Davies et al. (1991) recorded plaque indices, bleeding indices, and degree of dental irregularity of the front teeth in a group of four hundred and seventeen children either receiving or not receiving orthodontic therapy with occlusal variations and again after three years. There were significant reductions in the plaque and gingivitis scores on all dental surfaces between the baseline and three-year examination in the two groups of children. The children who received orthodontic treatment had a greater reduction, but this appeared to be related to behavioural factors rather than to improved dental alignment (Davies et al., 1991). Meaning that during the orthodontic treatment the oral hygiene was improved due to repetitive oral hygiene instructions.

I.3.c Different orthodontic materials

Most of the studies concerning the influence of bracket placement show microbial changes accompanied by increased signs of gingivitis after banding and bonding of fixed orthodontic appliances. The microbial and clinical

periodontal differences between bonding and banding teeth are not studied until now. The same can be said about the hundreds of commercially available bracket systems. As the design and the material characteristics of those orthodontic bracket types vary considerably (Anhoury et al., 2002), plaque adhesion and therefore the induction of gingivitis (Löe et al., 1965) might differ among currently used bracket versions. These microbial and periodontal differences between the different orthodontic materials are not thoroughly studied.

More information is available on the adhesion of *Streptococcus mutans* on different bracket materials, but most of these studies were performed in vitro with its known disadvantages. Brusca et al. (2007) compared the adhesion of *S. mutans* and *Candida albicans* on different brackets, also the association of both species was evaluated (Brusca et al., 2007). The adherence of Streptococcus mutans was not modified by the different brackets. The adherence of *C. albicans* was increased by the composite bracket, whereas the use of metallic brackets decreased the number of colony forming units (CFU). By electron microscopy they demonstrated that the adherence of *S. mutans* and *C. albicans* together varied according to the bracket material, with the highest adhesion on composite followed by ceramic and the metallic brackets demonstrated the lowest adhesion. Because of their results the authors proposed that the use of metallic brackets may be useful in a particular group of patients with a risk of *C. albicans* infection, such as patients with diabetes, immunosuppressive states, and periodontal disease (Brusca et al., 2007).

Apart from the bracket material, the way to attach the orthodontic wire to the brackets is a topic of interest. The wire can be attached with a metallic ligature wire, which is a very time consuming ligation method. Since 20 years, elastomeric ligatures are developed to speed up this procedure. These elastomeric rings have for long been considered to give increased bacterial counts. Recently in an in vivo study, different elastomeric ligatures were tested for their *S. mutans* adhesion. The Super Slick elastomeric rings (TP Orthodontics, LaPorte, Ind), which are especially developed to give less microbial adhesion, were tested versus conventional elastomeric ligatures. There was no clinical evidence that Super Slick elastomeric rings are effective in reducing bacterial biofilm formation on their surfaces and a recommendation

for their use in orthodontic therapy for that purpose is not justifiable (Magno et al., 2008).

Türkkakraman et al. (2005) conducted a study to follow the changes in microbial flora and periodontal status after orthodontic bonding to investigate whether two different archwire ligation techniques affect these changes (Türkkahraman et al., 2005). Although teeth ligated with elastomeric rings exhibited slightly higher numbers of microorganisms than teeth ligated with steel ligature wires, the differences were not statistically significant and can be ignored. The two archwire ligation techniques showed no significant differences concerning the gingival index, plaque index or pocket depths of the bonded teeth. However, teeth ligated with elastomeric rings were more prone to bleeding (Türkkahraman et al., 2005). These findings might lead to the conclusion that self-ligating brackets are less likely to promote microbial adhesion due to the absence of elastomeric rings. It has been postulated for example by the manufacturers that due to the smaller size and the absence of the elastomeric rings, self-ligating brackets accumulate less dental plaque than traditional twin brackets, resulting in fewer signs of gingival inflammation. Until recently, only a few case reports but no *de novo* plaque formation assays or randomized clinical trials with different orthodontic brackets have been reported (Alexander, 1991; Shelley, 1981; Zachrisson & Zachrisson, 1972).

I.4 Conclusions

From the literature we can conclude that the placement of orthodontic fixed appliances leads to increased microbial loads. In cases of good oral hygiene this might be controlled, but most studies indicate the development of generalized gingivitis during treatment (Alstad & Zachrisson, 1979; Davies et al., 1991; Huser et al., 1990; Naranjo et al., 2006; Sallum et al., 2004). Which is not likely to evolve into periodontitis, only few studies did report on attachment loss during treatment. A change in microbial environment responsible for the clinical manifestations after the placement of orthodontic brackets is evident, but not elucidated completely. For this purpose longitudinal studies in which subjects are monitored from bracket placement to bracket removal are needed. Furthermore evidence on the differences in periodontal and microbial reactions

between bands and brackets or different bracket types is completely not available. Until now most orthodontists do assume that the placement of orthodontic bands does provoke more adverse effects than it would be the case with brackets.

I.5 Aims

The aims of this research project were:

1. To elucidate the microbial (and periodontal) consequences of the placement of different orthodontic brackets and to compare the changes over time between the different bracket-types and with non bonded control teeth.
    a. *In vivo*, to make it possible to monitor the periodontal as well as the microbial changes.
    b. *In vitro*, to compare several different bracket-types

2. To follow up the short term microbial and periodontal changes during an orthodontic treatment (until 3 months after bracket placement). The banded and bonded teeth are to be compared to each other and to non-bonded control teeth.

3. To monitor the microbial and periodontal changes during an orthodontic treatment until bracket removal and to see whether or not the values do normalise 3 months after bracket removal.

4. To monitor the microbiological and clinical periodontal changes after placement of orthodontic attachments over a 1 year period and to link these changes to alterations in cytokine concentrations in the GCF. The hypothesis is stated that the cytokine concentrations prior to orthodontic treatment do have a predictive value for the clinical reaction during treatment.

CHAPTER II:

IN VIVO STUDY

INFLUENCE OF BRACKET DESIGN ON MICROBIAL AND PERIODONTAL PARAMETERS *IN VIVO*.

Aim: To compare undisturbed plaque formation on teeth bonded with different types of orthodontic brackets with non bonded control teeth, via a *de novo* plaque growth experiment over a 7-day period. **Material and Methods:** A randomized controlled trial with split-mouth design was set up enrolling 16 dental students. Within each subject sites with Speed® (S) and GAC® (G) brackets and control sites were followed. Clinical periodontal parameters were recorded at baseline, on day 3 and on day 7. Microbiological samples were taken from the brackets and the teeth on days 3 and 7. **Results:** Both anaerobe and aerobe colony forming units (CFU) were significantly higher in S- than G-sites ($P<.05$). The shift from aerobic to anaerobic species was observed earlier in S- than G-sites. The CFU ratio (aerobe/anaerobe) was significantly lower in S- than G-sites ($P<.05$). On day 3 the crevicular fluid flow was significantly higher in S- than control sites ($P<.05$). On day 7, S- and G-sites showed a significantly higher crevicular flow than control sites ($P<.05$). More hypertrophy was seen in S- than G- and control-sites ($P<.05$). No significant differences for bleeding on probing were observed. **Conclusions:** Bracket design can have a significant impact on bacterial load and periodontal parameters.

II.1 Introduction

Adverse reactions due to orthodontic treatment with fixed appliances have been described. Besides decalcification, leading to white spots and eventually caries (Benson et al., 2003; Benson et al., 2005; Chang et al., 1997; Glasspoole et al., 2001; VanMiller & Donly, 2003), periodontal harm may occur (Diedrich, 1989; Ellis & Benson, 2002; Wehrbein & Diedrich, 1992). One of the adverse periodontal reactions is a hyperplastic/ hypertrophic form of gingivitis which in our clinic is estimated to occur in one out of ten patients treated with fixed orthodontic appliances. This reaction might be elicited by a change in microbiological environment (Lee et al., 2005).

As the consequences for oral health are uncertain and since periodontal breakdown at young age could eventually put a burden on oral health long term, interruption of the orthodontic treatment is often advised when a hyperplastic/hypertrophic form of gingivitis is diagnosed.

It is well established that bacterial plaque is the primary etiological cause of gingival inflammation (Löe, 1965). As the design and the material characteristics of orthodontic bracket types varies considerably (Anhoury et al., 2002), plaque adhesion and therefore also the induction of gingivitis (Löe, 1965) might differ among currently used bracket types.

So far no *de novo* plaque formation assay has been set up with orthodontic brackets in the mouth. The same holds true for randomized controlled trials, as only case-reports considering few patients have been published (Alexander, 1991; Shelley, 1981).

The aim of this study was to compare the microbiological environment, the clinical periodontal parameters and the crevicular fluid flow between the Speed® and GAC® bonded teeth with control teeth by means of a *de novo* plaque growth experiment with a duration of 7 days. The hypothesis was that Speed® brackets, would show less bacterial colonization than traditional twin brackets, resulting in fewer signs of gingival inflammation.

II.2 Material and methods

II.2.a Subjects

Sixteen dental students from the Catholic University of Leuven (9 female and 7 male, Caucasians, aged between 17 and 27 yrs) participated (see Table II.1). They were given a written explanation of the background of the study, its objectives and their involvement. After screening for their suitability and after good comprehension of the protocol, they all gave their written informed consent. During the experiment the participants could always contact the researcher for questions or remarks.

Table II.1: General information on study population with data on previous orthodontic treatment, age and gender distribution.

		previous treatment with fixed appliance		age (years)	
	n	no	yes	mean	SD
male	7	2	5	20,8	0,8
female	9	3	6	21,0	2,6
total	16	5	11	20,9	1,9

The initial placement of the brackets was performed via a randomized protocol by means of concealed envelopes. The students were selected to fulfil the following inclusion criteria: no smoking, absence of extensive dental restorations or adhesive fixed partial dentures, a sulcus bleeding index (Muhlemann & Son, 1971) of less than 0,3 and no antibiotics during or up to 4 months prior to the study.

The students were also asked whether they already received an orthodontic treatment with fixed appliances, because this might have consequences for smoothness of the buccal enamel (Eliades et al., 2004) and as such on the microbial adhesion in the early formation of a dental plaque film (Quirynen et al., 1989; Quirynen et al., 1990). The design of this study was approved by the Ethical Committee of the Catholic University of Leuven.

II.2.b Experimental design

The study had a randomized, examiner blind, split-mouth design. In every student the mouth was divided into 4 quadrants, 2 of those served as controls. Two types of brackets were used, the Speed® (Strite Industries, Cambridge, Ontario, Canada) and the Generus bracket (GAC International, Bohemia, New York, USA) further mentioned as GAC®.

For the split-mouth comparison, twelve different sites were defined: the first and second premolars and the first molar of each quadrant. As one student had her first premolars extracted in the upper jaw, the canines were used instead.

The brackets were placed in contralateral antagonistic quadrants. The first quadrant used for bracket placement and the order in which the brackets were placed were randomly chosen by means of concealed envelopes, the second one was at the other side of the mouth in the antagonistic jaw. The GAC® (G-sites) and Speed® (S-sites) bonded teeth were alternated, giving rise to 4 different experimental settings.

The teeth bonded with the different brackets were compared with each other and with the non bonded control sites (Figure II.1). Prior to the study all patients received a professional scaling and polishing followed by an oral hygiene instruction to ensure a healthy periodontal situation. During the study period the students visited the clinic 3 times (Table II.2), the first time (on day 0) to record the *status praesens* of the periodontium (periodontal pocket depth (ppd) indicating the gingival overgrowth and/or swelling, the crevicular fluid flow, bleeding on probing (bop)) and to place the brackets. On the second visit (on day 3) the brackets were removed from the first premolars and the clinical measurements for these teeth were repeated. On day 7 the students came for the last clinical measurements, the removal of the last brackets and for a thorough cleaning and polishing of the teeth.

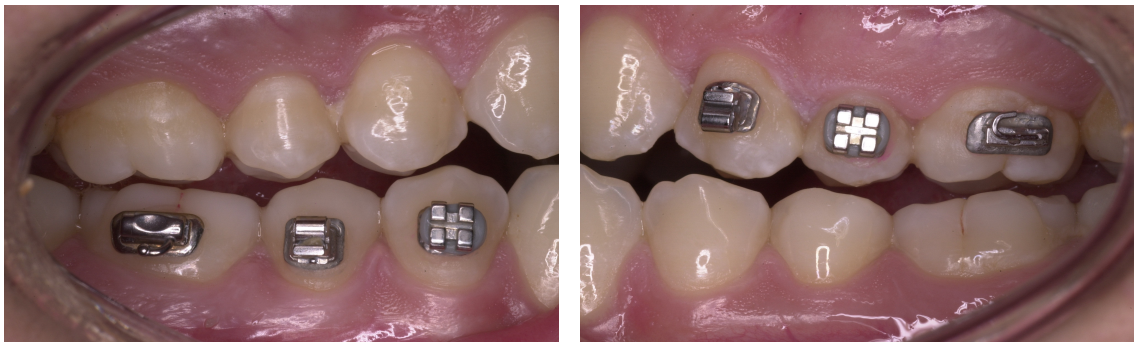

Figure II.1: Clinical example of the experimental setting on day 3 before plaque disclosure, with Speed® brackets bonded on teeth 24, 26 and 45; and GAC® brackets on teeth 25, 44 and 46.

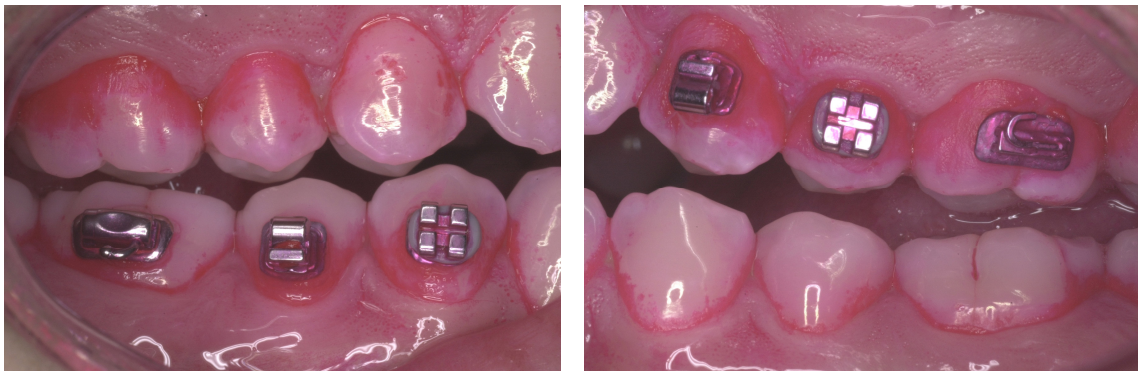

Figure II.2: Clinical example of the experimental setting on day 3 after plaque disclosure, with Speed® brackets bonded on teeth 24, 26 and 45; and GAC® brackets on teeth 25, 44 and 46.

Table II.2: Flow chart of the study.

	intake	day 0	day 3	day 7
scaling and polishing		x		x
crevicular fluid sampling		x	x	x
ppd measuring		x	x	x
bop measuring		x	x	x
bracket placement		x		
removal brackets PM1			x	
removal brackets PM2 and M2				x
oral hygiene instruction	x	x	x	x

On day 3 we compared 4 first premolars, one bonded with a Speed® bracket, one bonded with a GAC® bracket and two non-bonded control teeth. On day 7, the other brackets were removed and then we compared the S-sites (one second premolar bonded with a Speed® bracket and one molar bonded with a Speed® tube) and the G-sites (one second premolar bonded with a Speed® bracket and one molar bonded with a Speed® tube) to the control sites (two non-bonded premolars and two non-bonded molars).

After removal of the dental plaque at baseline all teeth were stained with erythrosine disclosing solution (4% erythrosine in water solution) to make sure there was no dental plaque remaining. From then on, the students were only allowed to clean the palatal and lingual surfaces of all teeth and the labial surfaces of their incisors with a single tufted brush. As such the undisturbed plaque formation could be followed over time.

II.2.b.1 Bracket placement/removal

The quadrants to bond were isolated with cotton-rolls and saliva suction. The enamel was etched locally with 37 % phosphoric acid for 30 seconds and rinsed rigorously with water afterwards. The etching was very accurately done by means of disposable mini sponge applicators (3M ESPE, St-Paul, USA) in order to avoid contact of the etching product with the gingiva. After rinsing with water excessively and drying, the sites were inspected for the characteristic dull, white, frosted appearance of adequately etched enamel. Re-etching never seemed necessary.

By means of the mini sponges, the primer (Concise™, 3M Unitek, Monrovia, USA) was applied in such way that it fully covered the etched enamel. Using these sponges made it easy to etch and prime only a little surface, which was fully covered by the bracket afterwards. The two components of bonding material (Concise™, 3M Unitek, Monrovia, USA) were mixed and directly applied to the bracket base. The bracket was pressed firmly onto the enamel surface and any excess of adhesive was removed. The brackets were positioned along the long axis of the teeth above the gingival margin and an attempt was made to place the brackets 3 mm from the gingival margin.

The involved teeth were stained with erythrosine to verify the baseline conditions since the primer might capture some pigment and give a pink shine after disclosure. Attention was paid in order to prevent the pink shine to be mistaken for dental plaque during the study.

After debonding, all the adhesive was removed from the teeth with a carbide bur (Komet, H282 204 010). After drying, the teeth were analyzed again for remaining adhesive. When the teeth were free of bonding material, they were cleaned with curettes, pumiced and received a fluoride application.

II.2.b.2 Microbiology

After isolating the teeth from saliva with cotton rolls and gently drying them to prevent contamination, the supragingival plaque was carefully removed by means of sterile curettes without traumatizing the gingiva as this would increase the production of crevicular fluid (Tanaka et al., 1998). The supragingival plaque was transferred into flip-capped vials containing 2.0 ml prereduced transport medium (RTF) to be processed (Syed & Loesche, 1972).

Each sample was homogenized by vortexing for 30 seconds and coded. The coding was not revealed until all analyses were completed, leading to blinded microbiological analyses.

The subgingival plaque was sampled after collecting the gingival crevicular fluid in order not to traumatize the crevice. Per site, six sterile medium paper points (RoekoA, Roeko, Langenau, Germany) were inserted (three mesially and three distally) and kept in place for at least 10s. The subgingival plaque samples were processed similar to the supragingival samples (van Gastel et al., 2007b).

Microbial samples were not taken at baseline for two reasons: first of all, there was only very little dental plaque available because of the good oral hygiene and secondly, the possible interindividual differences at the start of the experiment are erased by comparing different sites in one subject (the split-mouth protocol).

II.2.b.3 Crevicular fluid

The Periopaper® (#593525, Ora Flow Inc., Amityville, NY, USA) absorbent strips were used to collect crevicular fluid (Griffiths, 2003). The crevicular fluid

was sampled at the buccal crevice of the test and control teeth (baseline, day 3 and day 7 respectively) as depicted in Table II.2. Dental plaque was carefully removed without traumatizing the gingiva as this would increase the production of crevicular fluid (Tanaka et al., 1998). The presence of dental plaque has also been shown to have a marked effect on the recorded volume of crevicular fluid in the strip (Griffiths et al., 1992; Griffiths, 2003; Stoller et al., 1990). The samples were taken after isolating the sites from saliva with cotton rolls and gently drying to prevent contamination. Periopaper® strips were placed into the sulcus until light resistance was experienced (Griffiths, 2003). After keeping the strip in place for 30 seconds, the absorbed volume was measured with the Periotron® 6000 (Ora Flow Inc., Amityville, NY, USA). As environmental factors can affect the rate of fluid evaporation from paper strips (Tozum et al., 2004), the measurements were done within 5 seconds after removal of the strip from the crevice to minimize evaporation. Per tooth four strips were used.

II.2.b.4 Periodontology

At baseline, on day 3 and on day 7 (before and after plaque disclosing) digital colour photos were taken in order to follow the undisturbed plaque formation (Figures II.1 and II.2). At the same occasions the following parameters were scored (on day 3 only the first premolars and on day 7 the remaining teeth): crevicular fluid flow, probing depths and bleeding on probing (Table II.2).

The pocket depths were measured at the buccal sides of the teeth: both proximal and strictly buccal for the premolars and proximal, mesio-buccal, mid-buccal and disto-buccal for the molars. The pocket depths were measured with a Merrit B ® Probe (Hu-friedy, Chicago, IL) and rounded off to the nearest 0,5 mm. The bleeding tendency on probing (bop) for each of the above-mentioned sites per tooth was also registered, 20 seconds after probing the depth of the pocket (absent= 0, present= 1). These parameters were scored at day 0, day 3 and day 7 and the examiner was blinded for the previous scores.

II.2.b.5 Culture techniques

All samples were transferred to the laboratory and processed within two hours. Serial 10-fold dilutions were prepared in RTF. Dilutions of 10^{-2} to 10^{-4} were plated in duplicate by means of a spiral platter (Spiral Systems®, Inc.

Cincinnati, OH, USA) onto non-selective blood agar plates (Blood Agar Base II®, Oxoid, Basingstoke, England), supplemented with haemine (5 µg/ml), menadione (1 µg/ml) and 5% sterile horse blood.

After 7 days of anaerobic incubation (80% N_2, 10% CO_2 and 10% H_2) in an anaerobic chamber and 3 days of aerobic incubation at 37° C, the total number of anaerobic and aerobic CFU was counted. From these data, the CFU ratio (CFU aerobe/CFU anaerobe) was calculated. The number of dark pigmented colonies (black pigmented bacteria) on a non-selective anaerobic plate, containing approximately 100 colonies, was counted. From the black-pigmented bacteria in the plaque samples, every third colony was subcultured on a blood agar plate. After 48h of anaerobic incubation, the pure cultures were identified by means of a series of biochemical tests (including N-acetyl-*β*-D-glucosaminidase, *α*-glucosidase, *α*-galactosidase, *α*-fucosidase, esculine, indole and trypsin activity) in order to differentiate *P. gingivalis, P. intermedia* from other pigmented *Porphyromonas* and *Prevotella* species.

II.2.b.6 Scanning electron microscopy

In order to elucidate differences in surface characteristics between the two bracket types, Scanning Electron Microscopic (SEM) images with several enlargement factors were taken.

II.3 Statistics

A linear mixed model was fit to the data. Teeth nested within jaws and students were used as the experimental unit. This allowed to control for both inter-subject and intra-mouth variability. In order to ensure a correct interpretation of the P-values, residual values were tested for normality by means of a normal quantile plot. For those variables where the residuals were not normally distributed, a log-transformation was applied to the data.

ANOVA-tables were made to see whether there was an interaction effect between time and treatment and whether the separate material and day effects were significant. When the separate effects were significant without a significant interaction, group means were taken over all groups of the other factor for comparison. As such the averages were taken over more data in

order to give more power to the significance tests. When a significant interaction effect was found, all the groups were analyzed separately. Whether the groups were taken together or separately, their comparison was always corrected with the Tukey-Kramer correction for simultaneous hypothesis testing. The provided p-values are rounded off up to the first significant digit.

Moreover a distinction was made between the subjects with more and less than average formation of crevicular fluid on day 0. The statistical analyses were repeated on these two groups separately to see whether there was a significant difference in the reaction to the placement of brackets between both subgroups.

II.4 Results

II.4.a Microbiology

The lack of a significant interaction effect for CFU aerobe, CFU anaerobe and CFU ratio (aerobe/anaerobe) legitimated combination of the data on day 3 and day 7 (Table II.3). The numbers of aerobe and anaerobe CFU in supragingival plaque samples from the different sites showed significant material differences (Table II.3). S-sites in general allowed higher microbial colonization than G-sites. In Table II.3 the results are separately depicted per day and per material. S-sites showed significantly higher CFU aerobe than G-sites ($P<.05$) and both G and S-sites had higher values than control sites ($P<.05$). The CFU aerobe were significantly higher for all sites on day 7 when compared with day 3 ($P<.05$). Also for the CFU anaerobe, significant differences between the materials were seen (Table II.3). The interaction effect between day and site was borderline significant ($P=.1$).

In Table II.4 the data are depicted per day. On day 3, the CFU anaerobe were only significantly higher in the S-sites compared to control sites ($P<.05$). On day 7, more anaerobe species were seen and both S- and G-sites showed significantly higher values than the control sites ($P<.05$). Between S- and G-sites no significant differences for CFU anaerobe were seen, neither on day 3, nor on day 7.

Table II.3: Amount of colony forming units (CFU) and the CFU ratio (aerobe/anaerobe) per site, days combined. The first part displays the averages per site; the second part the differences between the sites with the corresponding p-values.

		CFUaerobe mean (SD)	P-value	CFUanaerobe mean (SD)	P-value	CFU ae/anae mean (SD)	P-value
averages per site	S-sites	1,20E+06 (1,89E+06)		2,20E+06 (1,92E+06)		0,57 (0,23)	
	G-sites	6,40E+05 (7,44E+05)		8,70E+05 (1,01E+06)		0,73 (0,21)	
	Control	2,70E+05 (4,84E+05)		4,10E+05 (6,67E+05)		0,62 (0,28)	
differences between sites	S-G	6.00E+05	<.05	1.30E+06	<.05	-0.16	<.05
	S-Control	9.60E+05	<.05	1.70E+06	<.05	-0.05	.54
	G-Control	3.70E+05	<.05	4.60E+05	<.05	0.12	<.05

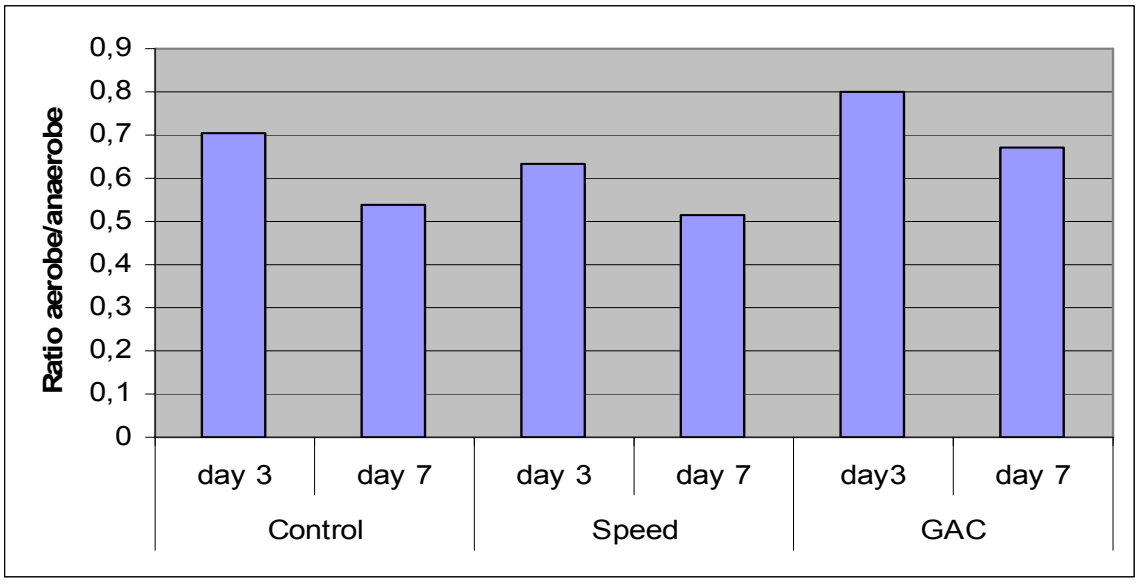

Figure II.3: Mean CFU ratio (y-axis) on the different sites depicted per day.

The CFU ratio (aerobe/anaerobe) was significantly lower for both S- and control sites, compared to G-sites (P<.05) (Table II.3 and Figure II.3). When the data per day were separated, there was only a significant difference for the S-sites compared to the G-sites (Table II.4). In this case, on day 7 the CFU ratio (aerobe/anaerobe) was significantly lower in the S-sites compared to G-sites (P<.05).

Table II.4: Amount of colony forming units (CFU) and the CFU ratio (aerobe/anaerobe) per site displayed per day. The first part displays the averages per site with the corresponding standard deviations; the second part the differences between the sites with the corresponding p-values.

			CFU aerobe		CFU anaerobe		CFU ae/anae	
averages per site	day	mat	mean	SD	mean	SD	mean	SD
	3	S-sites	1.23E+06	2.51E+06	1.93E+06	2.04E+06	0.64	0.38
		G-sites	4.62E+05	5.36E+05	5.77E+05	6.64E+05	0.80	0.27
		Control	1.84E+05	5.83E+05	2.26E+05	7.38E+05	0.70	0.46
	7	S-sites	1.23E+06	1.01E+06	2.40E+06	1.82E+06	0.51	0.41
		G-sites	8.80E+05	8.58E+05	1.31E+06	1.12E+06	0.67	0.36
		Control	3.94E+05	3.70E+05	7.28E+05	5.16E+05	0.54	0.37
differences between sites	day	mat	difference	P-value	difference	P-value	difference	P-value
	3	S-G	7.64E+05	.12	1.35E+06	<.05	-0.17	.58
		S-Control	1.04E+06	<.05	1.70E+06	<.05	-0.07	.96
		G-Control	2.78E+05	.16	3.51E+05	.08	0.10	.90
	7	S-G	3.53E+05	.80	1.09E+06	.25	-0.16	<.05
		S-Control	8.39E+05	<.05	1.68E+06	<.05	-0.03	.99
		G-Control	4.86E+05	<.05	5.81E+05	.28	0.13	.08

II.4.b Crevicular fluid

The crevicular fluid volume showed a significant material effect over time. Because of this significant interaction effect, the data of day 3 and 7 could not be combined. An overall increased crevicular fluid flow from day 3 to day 7 was obvious (Table II.5). On day 3 there was only a significantly higher flow in S-sites compared to control sites (P<.05), but on day 7 the flow was significantly higher in S- and G-sites versus control sites (both P<.05). The difference between S- and G-sites never reached a level of significance (day 3 P=.30, day 7 P= 1.00).

Table II.5: Periotron® read-outs per site displayed per day. The first part displays the averages per site with the corresponding standard deviations; the second part the differences between the sites with the corresponding p-values.

crevicular fluid volume									
	day	material	mean	sd		day	material	difference	P-value
absolute values	3	S-sites	31.58	16.00	difference between sites	3	S-G	8.73	.30
		G-sites	22.85	18.23			S-Control	12.23	<.05
		Control	19.36	9.22			G-Control	3.49	.93
	7	S-sites	38.10	15.28		7	S-G	-0.41	1.00
		G-sites	38.51	16.16			S-Control	12.83	<.05
		Control	25.27	10.89			G-Control	13.24	<.05

II.4.c Periodontology

On day 1 no significant intermaterial differences in ppd were present (P=.9997, P=.9999 P=1,00). The increase in probing depth (Table II.6) was significantly higher for S-sites compared to G- and control sites (P<.05). The latter was due to changes at the proximal sites (P<.05) since the mid-buccal sites showed no significant changes. The portion of sites bleeding on probing clearly increased over time for all sites (P<.05), but intersite differences were not detected (P=.44).

Table II.6: Increase in periodontal pocket depth (ppd) compared to day 1 in millimetres between the different sites, calculated over all the measurements (ppd-tot) and proximal (ppd-prox) sites.

	difference in ppd with day 1					
	materials	mean	SD	materials	difference	P-value
ppd-tot	S-sites	0.35	0.28	S-G	0,16	<.05
	G-sites	0.19	0.24	S-control	0,14	<.05
	Control	0.21	0.32	G-control	-0,02	.93
ppd-prox	S-sites	0.44	0.46	S-G	0.23	<.05
	G-sites	0.21	0.32	S-control	0.17	.09
	Control	0.27	0.40	G-control	0.06	.69

When a distinction was made between the subjects with more and less than average formation of crevicular fluid on day 1, no significant differences in the reaction on bracket placement for both the microbiological and clinical periodontal parameters were recorded. The same can be said about the subjects that received previous orthodontic treatment. No differences in undisturbed plaque growth were seen compared to the non-treated students.

II.4.d Scanning electron microscopy

These qualitative SEM images revealed remarkable irregularities on the interfaces between the different parts of the Speed® attachments (both of the bracket and the tube). These parts seem to be welded together causing an irregular surface, which might have lead to the increased plaque adhesion in the S-sites (Figure II.4).

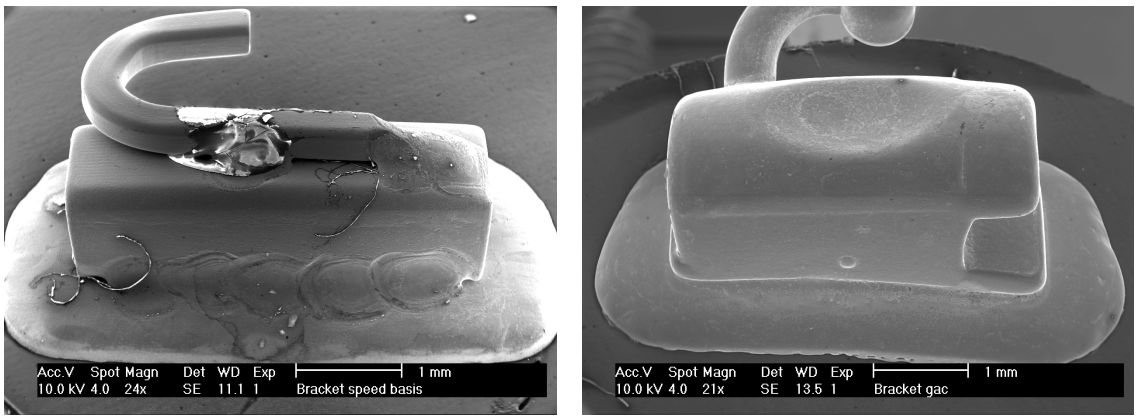

Figure II.4: Scanning Electron Microscopic (SEM) images of the different brackets, the Speed® bracket on the left and the GAC® bracket on the right.

II.5 Discussion

This *de novo* plaque growth experiment with split-mouth design succeeded in detecting some significant differences between the bonded teeth and the non bonded control teeth and also between the Speed® and GAC® bonded teeth. We could observe more significant differences between the different sites when all the means were combined and averaged over all days, not taking in account whether they were recorded on day 3 or on day 7. Of course, this is only statistically valid if no interaction effect is present. Depicting the data per day reduced the total number of measurements per category and therefore also the power of the statistical analysis.

The study was designed to make a valid comparison on day 3 and on day 7. On day 3 the 4 first premolars, one bonded with a Speed® bracket, one with a GAC® bracket and two non-bonded control teeth were compared. On day 7 the other brackets were removed and then the Speed® bonded teeth (one second premolar bonded with a Speed® bracket and one molar bonded with a Speed® tube) and the GAC® bonded teeth (one second premolar bonded with a GAC® bracket and one molar bonded with a GAC® tube) were compared with the control teeth (two non-bonded premolars and two non-bonded molars). By combining a bracket and a tube of one system, we compensated the fact that a molar tube has a different design and a bigger surface than a premolar bracket.

The use of dental students with good oral health as subjects for this study is important as several studies indicated an increased plaque accumulation in the presence of gingival inflammation (Quirynen et al., 1991; Rowshani et al., 2004). This increased plaque formation has also been shown in experimental gingivitis studies (Daly & Highfield, 1996; Quirynen et al., 1991). As we were most interested in the early dental plaque formation, the duration of the study was set at 7 days. This was also positive for the compliance of the dental students as longer periods would have resulted in a decrease in compliance of non-brushing. It was rather easy to verify the presence of undisturbed dental plaque formation: first of all the regularity of the biofilm on the bonded teeth could easily be observed. Secondly, the tooth mesial of the teeth under investigation served as control (in most cases the canine); when the

dental plaque on the buccal surface of this tooth was not disturbed: the more distal teeth were supposed to be untouched.

To ensure blind evaluation, the measurements on day 1 were done prior to the randomization (before opening the concealed envelopes). On days 3 and 7 the measurements were done after removal of the brackets so that blind measuring was ensured again. The researcher did not know which bracket was bonded on which tooth. All laboratory analyses were done with an unknown coding system, which was only revealed after the completion of the study.

The increased probing depth recorded during this study is most likely caused by gingival enlargement or by deeper penetration of the probe into the weakened junctional epithelium (Anderson et al., 1991; Jansen et al., 1981). As these two processes could simultaneously contribute to the increase in probing depth, a distinction between these processes cannot be made with the instruments used in this study During the relatively short period of this study, gingivitis was induced, but attachment loss probably did not occur (Naranjo et al., 2006).

The increase in crevicular fluid described before, is unlikely to be induced by the procedure of bracket placement alone. Phosphoric acid is widely used as an etching product in both adhesive dentistry and orthodontics but necrotizing effects on periodontal soft tissues have been reported (Forsberg, 1982). White ulcerative lesions were described in cases where the phosphoric acid was accidentally in contact with the mucosa for several minutes (Akman et al., 2005). To avoid these adverse effects in this study, the phosphoric acid was applied locally on a safe distance from the gingival margin with a little non soaked mini-sponge. After 30 seconds the tooth was excessively rinsed with water.

Dental adhesives can have adverse effects on the gingiva too. They are toxic to the gingival fibroblasts *in vitro* (Gurpinar et al., 2006; Huang et al., 2002). Particularly the residual monomers may cause gingival inflammation and irritation (Gioka et al., 2005). The same gingival contact prevention protocol as used for the phosphoric acid was applied for the bonding material. The bonding material was directly polymerized and thus contact with the gingival margin was prevented. These were the reasons why we assumed that these two products did not contribute to the inflammatory response seen in this *de novo* plaque

growth experiment. Etching and bonding of the control teeth was not considered because of this prevention protocol and the fact that we were interested in the effects of bracket placement, compared to non-treated (non bonded) teeth.

Besides the crevicular flow, the bleeding on probing tendency is an other parameter to quantify gingival inflammation and was significantly higher on day 7 compared to baseline for all sites. There were however no significant intersite differences detected on the different days. A possible explanation for the lack of significant intermaterial differences was possibly due to the lower discriminative value of this parameter.

Comparison of the bonded teeth to the non-bonded control teeth showed a faster undisturbed plaque formation in the bonded sites, 2 till 8 times more CFU were counted (Table II.3 and II.4). These results are consistent with most changes in crevicular fluid flow and the periodontal pocket depth measurements (Table II.5 and II.6). Concerning the crevicular flow, neither on day 3 nor on day 7 significant differences between the two bracket types were seen. But the higher bacterial load and the lower CFU ratio (aerobe/anaerobe) at the S-sites might have resulted in a faster increase in crevicular fluid flow there. The crevicular flow at the S-sites was already significantly higher than the control sites on day 3, whereas the G-sites did not show higher crevicular flows compared to the control sites earlier than day 7.

A significantly higher crevicular flow in the S-sites versus control sites on day 3 ($P<.05$) was seen without a significant difference in CFU ratio (aerobe/anaerobe) between these two groups. Nevertheless there was a significant increase in absolute values of both aerobe and anaerobe CFU, which could be the explanation. On day 7, the G-sites showed significantly higher crevicular flows than the control sites, without a significantly lower CFU ratio (aerobe/anaerobe) and without significantly higher anaerobe CFU.

The fact that the CFU ratio (aerobe/anaerobe) was lower in the control sites than in the G-sites was another unexpected outcome. A possible explanation is that the dental plaque retrieved from the control sites is mostly formed in strict contact with the gingival margin as well as the interdental space (Figure II.2). These two niches are predominantly colonized by anaerobe bacteria. The total amount of bacterial plaque however was higher on G-sites than on the control teeth. This was expected as the total surface available for

plaque adhesion is significantly larger and more irregular at the G-sites. The natural automatic cleaning of the teeth by food mastication is not or less present with brackets in place. The lower CFU ratio (aerobe/anaerobe) as mentioned above was not supported by the crevicular fluid flow which was lower in the control sites. This implies that in this experiment, not the CFU ratio (aerobe/anaerobe) alone is responsible for the development of gingivitis, but also the total amount (absolute values) of bacteria.

Significant differences were seen for the increase in pocket depth (Table II.4), CFU aerobe, CFU anaerobe and the CFU ratio (aerobe/anaerobe) (Table II.5). The increased CFU counts in the S-sites compared to the G-sites were not expected because of the limited dimensions of the Speed® bracket and the presence of a smooth clip instead of an elastomeric ligature. To find differences in surface characteristics between the two bracket types, Scanning Electron Microscopic (SEM) images with several enlargement factors were taken. These qualitative SEM images revealed remarkable irregularities on the interfaces between the different parts of the Speed® attachments (both of the bracket and the tube). These parts seem to be welded together causing an irregular surface, which might have lead to the increased plaque adhesion in the S-sites (Figure II.4).

Because randomized controlled trials of this type have not been performed so far, it is not possible to compare our findings to those of other authors. Different orthodontic bracket types have not been compared microbiologically and clinically *in vivo* yet. Nevertheless there are indications that placement of orthodontic fixed appliances has an impact on the microbiological characteristics of the dental plaque. Lee et al. (2005) found significant differences in the prevalence of putative periodontal pathogens in subgingival dental plaque from gingivitis lesions in orthodontic patients. Their study succeeded in detecting significant differences between the subgingival dental plaque of gingivitis lesions in patients with and without orthodontic fixed appliances. *T. forsythia, T. denticola, and P. nigrescens* were significantly more common in the samples obtained from the orthodontic patients than in the samples obtained from the non-orthodontic control patients (Lee et al., 2005). Their results as well as ours indicate that the local changes associated with the wearing of orthodontic brackets may affect the prevalence of periodontal

pathogens in dental plaque. However, no differences in periodontal pocket depth were seen between their groups and no different bracket types were evaluated. Also the orthodontic group might have received more hygiene instructions resulting in a better oral hygiene, making the two groups difficult to compare.

Huser et al (1990) performed clinical and bacterial examinations before the beginning of treatment and after placement of the orthodontic appliances up to 90 days. Plaque index and bleeding scores increased significantly on banded teeth as compared with control sites, the probing depth however remained within normal values for both test and control groups. The composition of dental plaque was only determined by dark-field microscopy and showed significant shifts in the test sites after banding, with an increased percentage of spirochetes, motile rods, filaments and fusiforms. During the same period no significant changes in the bacterial distribution were observed in the control group (Huser et al., 1990). Petti et al (1997) performed a similar study with a duration of 6 months. They compared the influence of fixed appliances with the influence of removable orthodontic appliances on supra and subgingival microbiota. Periodontal parameters were not examined. Their data suggest that in well motivated patients keeping up with the oral hygiene, gingivitis and periodontitis did not occur during the first six months of treatment. The significant modification of oral microbiota, shown by subjects with fixed appliances however, is in line with our findings and suggests that the risk for gingivitis during the following months of therapy is still high and the risk for periodontitis cannot be excluded (Petti et al., 1997).

Regardless of the level of plaque control, many subjects undergoing treatment with fixed orthodontic appliances develop generalized gingivitis within a short time (Alexander, 1991; Kloehn & Pfeifer, 1974; Sallum et al., 2004; Zachrisson & Zachrisson, 1972). The qualitative change in the microbiota, which involves the growth of periodontopathogenic bacteria, could be associated with the gingival inflammation around the orthodontic brackets. Different orthodontic bracket materials could therefore have dissimilar clinical manifestations.

II.6 Conclusion

It is clear that significant differences concerning the microbial adhesion were detected. These differences were accompanied by similar trends in GCF flow and probing depth. This indicates that the variation in bracket design has repercussions on the clinical reaction of the patient undergoing orthodontic treatment. Further research should be performed to visualize the potentially different microbial/ periodontal complications of different orthodontic bracket systems used in treatment with fixed appliances, in such way that brackets can be designed to reduce plaque adhesion.

CHAPTER III:

IN VITRO STUDY

MICROBIAL ADHESION ON DIFFERENT BRACKET TYPES *IN VITRO*.

Aim: To test the hypothesis that there are differences in the total bacterial counts and capacity for biofilm formation between 7 different bracket types. **Material and Methods:** By means of an in vitro experiment, 7 commercially available bracket systems, Damon (A), Clarity (B), Mystique (C), Speed (D), Victory MBT (E), Micro-loc (F) and Generus (G) were compared. 25 premolar brackets of each bracket system were incubated in brain heart infusion medium containing the saliva and bacteria of two orthodontic patients. After 72 hours, the amount of aerobe and anaerobe bacteria was determined by counting the colony forming units (CFU). The CFU ratio (aerobe/anaerobe) was also calculated and the black pigmented bacteria were analyzed. **Results**: Significant differences between the different bracket types concerning biofilm formation are found. The bracket types can be arbitrarily divided into low, intermediate and high plaque retaining brackets. The group with low adhesion consists of bracket types E, F and G, the group with high adhesion of bracket types A, B and C, and type D exhibits intermediate adhesion. The group with high microbial adhesion (A, B and C) did present significantly lower CFU ratios (aerobe/anaerobe) than the other bracket systems ($P<.05$). **Conclusions:** The hypothesis is accepted. Orthodontic brackets serve as different loci for biofilm formation and in this in vitro study there were significant differences between the different types of brackets.

III.1 Introduction

Despite the widespread use of fixed orthodontic appliances, there is only little scientific evidence available on the microbial implications of the different bracket systems *in vivo*.

In the previous chapter a randomized clinical trial with split-mouth design was presented. Significant differences, *in vivo,* between two different bracket systems were detected (van Gastel et al., 2007b). The observed microbiological changes and significant differences between the two bracket types were confirmed by the gingival crevicular fluid flow rates and the periodontal pocket depths.

Because of the relation between the microbiological profiles of the different bracket types and the clinical reaction, it became of interest to compare biofilm formation on different bracket types by means of an *in vitro* study.

The aim of the present investigation was to compare early microbial adhesion on different brackets in vitro, in the same circumstances as they are used clinically.

III.2 Material and methods

III.2.a Brackets

Seven commercially available orthodontic brackets as shown in Table III.1 were used in this study.

Table III.1: Information about the different bracket systems used in this experiment: the commercial names, manufacturer, composition, for which orthodontic technique they are used and whether or not these brackets need to be ligated by means of an elastomeric ring.

	bracket	manufacturer	material	technique
A	Damon 3	Ormco, Orange, California, USA	metal	self-ligating
B	Clarity	3M Unitek, Monrovia, California, USA	ceramic	straight wire
C	Mystique	GAC International, Bohemia, New York, USA	ceramic	straight wire
D	Speed	Speed System Orthodontics, Canada	metal	self ligating
E	Victory MBT	3M Unitek, Monrovia, California, USA	metal	straight wire
F	Micro-Loc	GAC International, Bohemia, New York, USA	metal	straight wire
G	Generus	GAC International, Bohemia, New York, USA	metal	full edgewise

These 175 brackets were maxillary premolar brackets: bracket types A to F had the Roth prescription and a 0.022-inch slot and G had a 0.018-inch slot without any prescription. All but the self-ligating brackets were ligated with elastomeric rings (Ormco, Glendora, California, USA) by the same person.

The brackets were placed randomly in a poly-urethane box on a grid with an inter bracket distance of 10 mm (Figure III.1). Before placing the brackets, the floor of the box was roughened with a diamond-coated burr at the exact places to bond the brackets, in such a manner that these areas were completely covered by the bracket bases. The brackets were then bonded with a composite bonding material (Transbond™ Plus colour change adhesive, 3M Unitek, Monrovia, California, USA) that changes colour from pink to white during the light-curing process. The composite was applied to the bracket base in order to cover the entire mesh and the bracket was pressed firmly onto the box. The excess of adhesive was removed, which was facilitated by its pink colour. Then the composite was light-cured (Dentsply QHL75 halogen curing light, Dentsply, Addlestone, Surrey, UK) for 30 seconds from both sides. The bracket placement procedure was done by one researcher (JvG) under magnification (2.5 x).

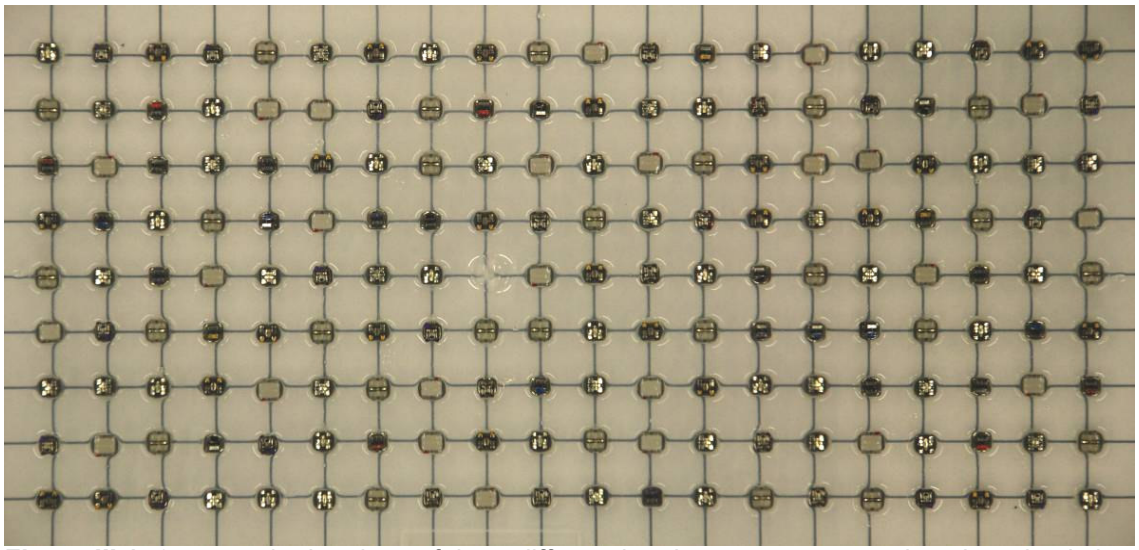

Figure III.1: 25 premolar brackets of the 7 different bracket systems were placed randomly in a poly-urethane box with a grid with an inter bracket distance of 10 mm.

III.2.b Medium

Supragingival plaque of two patients wearing full bonded appliances was collected directly before the start of the experiment by means of sterile curettes and was transferred into a sterile flip-capped vial containing 2 ml RTF (Syed & Loesche, 1972). Saliva was collected too from these two patients, who had refrained from eating, drinking and brushing for at least 2 hours before saliva collection. They had no acute dental caries or periodontal lesions and had neither received professional cleaning nor antibiotic medication in the 3 months before the study. Per patient 5 ml unstimulated whole saliva was collected in a chilled sterile tube by the spitting method. The dental plaque and the saliva were added to one litter of Brain Hearth Infusion (BHI) resulting in a concentration of $8.0*10^3$ CFU aerobe/ml and $1.4*10^4$ CFU anaerobe/ml.

The BHI medium containing the bacteria and saliva of the orthodontic patients was inserted into the container carrying the brackets and was incubated in an aerobe oscillating incubator for 72 hours at 37 °C and at 40 rpm.

III.2.c Bracket removal

The medium was removed from the container and microbiologically analysed. The concentration of bacteria in the medium at removal was $8.3*10^6$ CFU aerobe/ml; $8.1*10^6$ CFU anaerobe/ml and $5.3*10^5$ CFU *P. intermedia*/ml. The experimental setting was then washed with saline 2 times in order to remove all non-adhered bacteria.

The brackets were removed randomly with a sterile pair of pliers and transferred into a flip-capped vial containing 1ml of prereduced transport medium (RTF) and coded (Syed & Loesche, 1972). The code used was not revealed, leading to a blind microbiological analysis. Just prior to diluting the samples, all bacteria were removed from the brackets by sonication for 15 minutes. The samples were then homogenized by vortexing for 30s and processed in less than 15 minutes by preparing serial 10-fold dilutions in RTF.

III.2.d Culture techniques

Analysis of the microbial samples is described in II.2.b.5.

III.3 Statistics

All data were log10-transformed. An Anova-model followed by Tukey multiple comparisons procedure was used to find significant differences between bracket types. The bracket types were divided into groups of high, intermediate and low bacterial adhesion. Between the groups significant differences were seen, while within one group significant differences were not detected. P-values of the latter were corrected for simultaneous hypothesis testing according to Tukey-Kramer. P-values below 0.05 were considered. For the analysis of *P. intermedia*, means and variances were estimated via a maximum likelihood model, taking into account measurements below detection limit. Residual analysis showed the presence of two outliers, which have been removed (one for bracket type B, one for type G)

III.4 Results

II.4.a Microbiology

III.4.a.1 CFU aerobe (Table III.2, Figure III.2.A)

Bracket types A, B, C and D did show significantly higher aerobe CFU counts than bracket types E, F and G (P<.05). Types A, B, C and D did not differ significantly from each other concerning the aerobe CFU. Within the group of bracket types with low bacterial adhesion (E, F and G), there was a ranking: G showed significantly lower aerobe CFU counts than E (P<.05) and F was positioned in between, without being significantly different from type E or G.

III.4.a.2 CFU anaerobe (Table III.2, Figure III.2.A)

Bracket types A, B and C did exhibit the highest anaerobe CFU counts. Bracket types E, F and G showed the least microbial adhesion. Bracket type D has an intermediate value for CFU anaerobe and shows significantly higher values than types E, F and G (P<.05). D showed significantly lower anaerobe CFU counts than A, B and C (P<.05).

III.4.a.3 CFU ratio (Table III.2, Figure III.2.B)

For the CFU ratio (aerobe/anaerobe) the bracket types A, B and C showed the lowest ratio and thereby probably most unfavourable biofilm. Within this group there is only a significant difference between A and B (P<.05), with lower values for B.

Bracket types E, F and G formed the group with significantly higher CFU ratios than the rest (P<.05), with G showing a significantly higher ratio than E (P<.05). Type D was situated between the group with the higher and the group with the lower ratio and differed significantly from both groups (P<.05).

III.4.a.4 CFU P. intermedia (Table III.2, Figure III.2.A)

The bracket types A, B and C showed a high detection frequency of *P. intermedia.* The CFU *P. intermedia* counts of bracket types A, B and C were significantly higher than the other types (P<.05), but there were no significant differences between them. Bracket types E, F and G showed the lowest CFU *P. intermedia* counts and did not differ significantly intra-group. Bracket type D did exhibit significantly lower *P. intermedia* counts than A, B and C and significantly higher counts than E, F and G (P<.05).

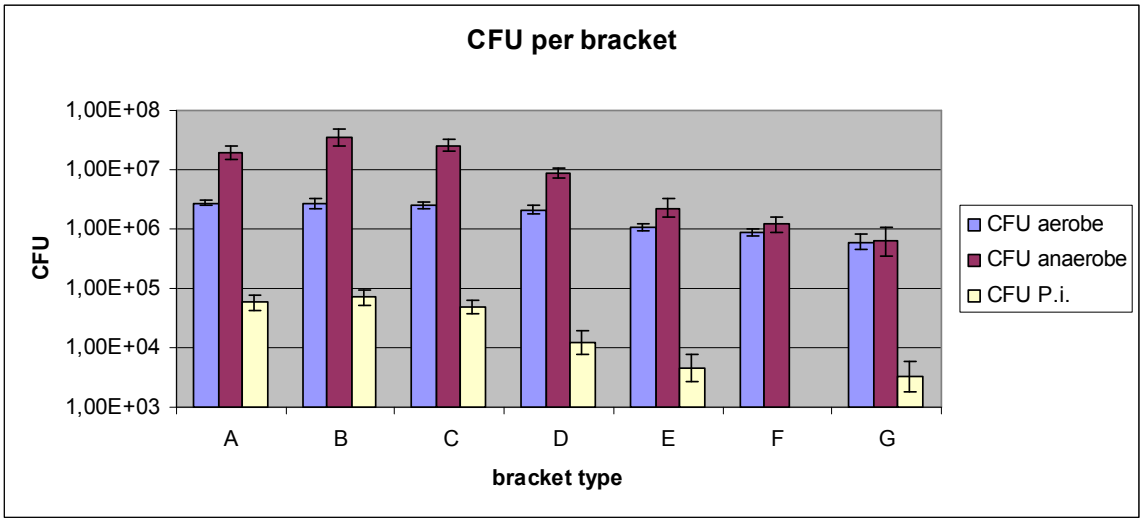

Figure III.2.A: Colony-forming units (CFU)/bracket for the different bracket types, with a logarithmic scale. Values are displayed as the mean and the 95% confidence interval. Damon (A), Clarity (B), Mystique (C), Speed (D), Victory MBT (E), Micro-loc (F) and Generus (G).

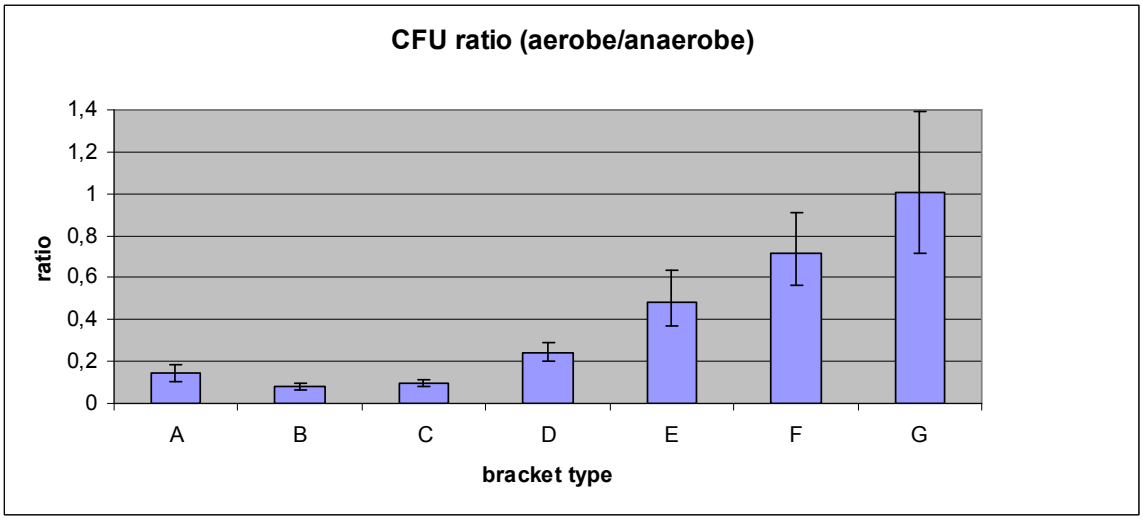

Figure III.2.B: CFU ratio (aerobe/anaerobe) for the different bracket types. Values are displayed as the mean and the 95% confidence interval. The lower the CFU ratio (aerobe/anaerobe) the more pathogenic the microfilm. Damon (A), Clarity (B), Mystique (C), Speed (D), Victory MBT (E), Micro-loc (F) and Generus (G).

Table III.2: Comparisons between the different bracket types for the aerobe colony-forming units (CFU), the anaerobe CFU, the *Prevotella intermedia* CFU and the CFU ratio (aerobe/anaerobe). The values are depicted as the ratio between two bracket types and the corresponding P-values. Damon (A), Clarity (B), Mystique (C), Speed (D), Victory MBT (E), Micro-loc (F) and Generus (G).

comparison	CFU ae	P-value	CFU anae	P-value	CFU P.i.	P-value	CFU ratio (ae/anae)	P-value
B/A	0.9667	1	1.8122	.13	1.2820	.9989	0.5506	.001
C/A	0.8082	.98	1.3418	.86	0.8704	1	0.6794	.27
D/A	0.5401	.15	0.4515	.01	0.2664	.1513	1.6963	.04
E/A	0.1159	<.0001	0.1151	<.0001	0.0928	.0006	3.4072	<.0001
F/A	0.0685	<.0001	0.0618	<.0001	0.0073	<.0001	5.0548	<.0001
G/A	0.0376	<.0001	0.0318	<.0001	0.0532	<.0001	7.0974	<.0001
C/B	0.8362	.99	0.7406	.8427	0.6789	.9868	1.2340	.8888
D/B	0.5596	.22	0.2492	<.0001	0.2078	.0455	3.0811	<.0001
E/B	0.1199	<.0001	0.0635	<.0001	0.0724	.0001	6.1887	<.0001
F/B	0.0709	<.0001	0.0341	<.0001	0.0057	<.0001	9.1812	<.0001
G/B	0.0389	<.0001	0.0175	<.0001	0.0415	<.0001	12.8914	<.0001
D/C	0.6694	.65	0.3364	.0001	0.3061	.2613	2.4963	<.0001
E/C	0.1434	<.0001	0.0858	<.0001	0.1066	.0017	5.0153	<.0001
F/C	0.0848	<.0001	0.0460	<.0001	0.0084	<.0001	7.4405	<.0001
G/C	0.0465	<.0001	0.0237	<.0001	0.0611	.0001	1.4448	<.0001
E/D	0.2142	<.0001	0.2550	<.0001	0.3484	.5011	2.0091	.001
F/D	0.1267	<.0001	0.1368	<.0001	0.0275	.0028	2.9806	<.0001
G/D	0.0695	<.0001	0.0704	<.0001	0.1996	.0999	4.1841	<.0001
F/E	0.5913	.32	0.5365	.1	0.0788	.0952	1.4835	.2519
G/E	0.3243	.0002	0.2761	<.0001	0.5729	.9706	2.0826	.001
G/F	0.5484	.2	0.5146	.06	7.2661	.3498	1.4038	.45

III.5 Discussion

A pilot study indicated that the presence of saliva in the medium was necessary in order to give adhesion of the oral bacteria to the bracket surfaces. Saliva of the patient donating the dental plaque was used in order to have an optimal interaction between the salivary components and the micro-organisms, which is recognized as the primary step in biofilm formation and associated diseases (Walker & Sedlacek, 2007). All brackets were placed in one recipient in order to ensure the same environmental circumstances for each bracket. Because of the random placement of the brackets, differences in shear forces due to the medium could not contribute to differences in microbial adhesion between the bracket types. The experimental setting as used in this study is not a common way to grow anaerobe biofilms. The oscillating incubator and the poly-urethane box do not provide an anaerobic environment and BHI is certainly not the first choice medium to grow anaerobes. We wanted to simulate the aerobic oral environment and this probably underestimates the amount of anaerobe species, but this will be true for all bracket types. The fact that we did find anaerobe species (*P. intermedia*) will most likely be due to the mutual protective environment between the different species in the multi-species biofilm. The same process makes it possible to find anaerobic species in supragingival dental plaque. We did not identify the different aerobic species present in the microbial samples although these are responsible for the initial biofilm formation, especially in trials with such short duration, these species are of great importance. But in the *in vivo* RCT (Chapter II), we clearly saw increased signs of inflammation and an increased pathological microbial environment at the bonded sites after 3 days (van Gastel et al., 2007b), which shows that the biofilm is already shifted to more anaerobe and more pathogen species and the anaerobes are the ones we are mostly interested in.

To ensure blind evaluation, all laboratory analyses were done with an unknown coding system, which was only revealed after the completion of the study. Elastomeric rings are said to promote bacterial adhesion compared to metal ligatures (Türkkahraman et al., 2005). In order to compare the brackets in a fair way, the non self-ligating brackets were ligated with an elastomeric ligature because this is most comparable to the clinical situation. The results of

this study indicate a significant difference between the different brackets concerning the adhesion of supragingival oral microbes. The bracket types can be arbitrarily divided in groups with low, intermediate and high microbial adherence. Bracket types E, F and G showed low microbial adhesion, bracket types A, B and C a high adherence, and type D is intermediate. The bracket types presenting high microbial adhesion also showed lower CFU ratios (aerobe/anaerobe) and higher CFU *P. intermedia* counts, probably due to a thicker and more mature biofilm leading to a more anaerobe environment at the base of this biofilm. *P. intermedia* is known to be one of the species that are elevated in orthodontic patients compared to non-orthodontic controls (Naranjo et al., 2006).

In our study, both ceramic brackets (E and F) were in the high microbial adhesion group and displayed the lowest CFU ratios (aerobe/anaerobe), this is in congruence with the findings of some researchers (Ahn et al., 2007; Brusca et al., 2007). Others did not find significant differences between brackets manufactured from different materials (Anhoury et al., 2002). Eliades et al. studied the basic principles of bacterial adhesion (Eliades et al., 1995): they compared the wettability of and the composition of salivary films on orthodontic raw bracket materials by contact angle measurements. Stainless steel presented the highest critical surface tension and total work of adhesion, indicating an increased potential for micro-organisms to attach on metallic brackets. These findings are not in line with ours, as we saw significantly higher adhesion on the ceramic brackets compared to the metal ones.

Anhoury et al. (2002) studied thirty-two metallic brackets and 24 ceramic brackets from orthodontic patients at the day of debonding and showed no significant differences between metallic and ceramic brackets with respect to the caries-inducing *S. mutans* and *L. acidophilus* counts. Mean counts of 8 of 35 additional species differed significantly between metallic and ceramic brackets, with no obvious pattern favouring one bracket type over the other (Anhoury et al., 2002).

Until now, only a few reports are available on the microbial alterations after placement of different bracket systems in vivo. Türkkahraman et al. (2005) conducted a study to investigate the differences in microbial flora and periodontal status between two different archwire ligation techniques

(Türkkahraman et al., 2005). These authors observed that although teeth ligated with elastomeric rings exhibited slightly higher numbers of microorganisms than teeth ligated with steel ligature wires, the differences were not statistically significant and thus can be ignored. Moreover the two archwire ligation techniques showed no significant differences concerning the gingival index, plaque index or pocket depths of the bonded teeth. However, teeth ligated with elastomeric rings were more prone to bleeding (Türkkahraman et al., 2005).

In our study the brackets ligated with an elastomeric ring did not per se show higher bacterial counts compared to the self-ligating brackets. For the metallic brackets (A, D, E, F and G) we can even conclude that the self-ligating brackets showed significantly higher ($P<.05$) bacterial counts and a lower CFU ratio (aerobe/anaerobe) than the classic brackets with elastomeric rings in this in vitro study. Scanning Electron Microscopic (SEM) images with several enlargement factors revealed remarkable irregularities on the interfaces between the different parts of the self ligating brackets (van Gastel et al., 2007b). These parts seem to be welded together causing an irregular surface, which might have lead to the increased biofilm formation on the self ligating brackets.

The results of this present study are in congruence with those of our previous study (van Gastel et al., 2007b). Van Gastel et al. (2007) compared the plaque formation on teeth bonded with bracket types D and G by means of a RCT with a split mouth design with non bonded control teeth. In that study both the anaerobe and aerobe colony forming units (CFU) were significantly higher in teeth bonded with bracket D than teeth bonded with bracket G ($P<.05$). The shift from aerobe to anaerobe species was observed earlier in teeth bonded with bracket D than teeth bonded with bracket G. The aerobe/anaerobe CFU ratio was also significantly lower in teeth bonded with bracket D than teeth bonded with bracket G ($P<.05$). These differences were also visible in the clinical periodontal parameters. In the present study the D brackets did also show significantly higher CFU counts (except for CFU *P. intermedia*) and lower CFU ratios than the G brackets.

III.6 Conclusions

Orthodontic brackets serve as different loci for biofilm formation and in this in vitro study there were significant differences between the different types of brackets. The ceramic brackets were both in the high adhesion group while the conventional twin brackets did present the least microbial adhesion. The self-ligating brackets were in the high and intermediate adhesion group, meaning that the elastomeric ligature was not overshadowing the impact of the bracket base.

The results from this in vitro study can not be extrapolated to an in vivo setting. Further comparisons by means of a RCT with several different bracket types could fill the lacuna in this field of clinical orthodontics.

CHAPTER IV:

SHORT TERM CHANGES

SHORT TERM MICROBIAL AND PERIODONTAL CHANGES DURING TREATMENT WITH FIXED ORTHODONTIC APPLIANCES

Aim: To monitor the longitudinal microbial and clinical periodontal changes after the placement of fixed orthodontic appliances and to compare the banded and bonded sites to control sites. **Materials and methods:** This longitudinal trial (split-mouth design) enrolled 24 patients. Microbiology (sub- and supragingival), probing depth, bleeding on probing and gingival crevicular fluid flow were assessed at baseline (band placement), and at weeks 18 (bracket bonding) 20, 24 and 36. A statistical comparison was made over time and between the banded, bonded and control sites, respectively. **Results:** Sub- and supragingival CFU ratio (aerobe/anaerobe) decreased significantly (relatively more anaerobes) over the period of the study for both the banded and the bonded sites ($P<.05$). This decrease was accompanied by significant elevations in probing depth, bop and GCF. These changes occurred faster after bonding, compared to banding. 18 weeks after banding no significant changes were observed except for the increased probing depth ($P<.05$). At week 36, all microbial and clinical parameters at the bonded site changed significantly in the negative direction ($P<.05$) compared to week 18. **Conclusions:** Placement of fixed orthodontic appliances has a significant impact on both the microbial and clinical parameters. The changes occurred faster at the bonded sites compared to the banded sites.

IV.1 Introduction

It is well established that bacterial plaque is the primary etiological factor in the development of gingival inflammation (Löe, 1965). The quantity as well as the quality of plaque are since long known to be influenced by many factors including surface characteristics (Quirynen et al., 1988; Quirynen et al., 1989; Quirynen et al., 1990). Besides the total amount of bacteria, also the ratio between the aerobic and anaerobic bacteria is an important marker for plaque pathogenicity (Petti & Renzini, 1994). The placement of orthodontic bands and brackets might influence the biofilm formation and maturation due to all above mentioned factors. Lee et al (2005) reported significantly higher prevalences of putative periodontal pathogens in subgingival plaque from gingivitis lesions in orthodontic patients compared to non-orthodontic patients (Lee et al., 2005). Most studies reporting on gingival changes after bracket placement noted only reversible periodontal changes (Gomes et al., 2007; Thomson, 2002). Others however, reported a significant loss of attachment during the orthodontic treatment (Janson et al., 2003).

The aim of this study was to monitor the microbial (both supra- and subgingival) and clinical periodontal changes after the placement of brackets and bands over time with short intervals and to compare these results, intra-subject, with non-bonded control teeth.

IV.2 Material and methods

IV.2.a Subjects

Twenty-four subjects (10 males, 14 females) aged 14.6 +/- 1.1 years [mean +/- SD]; referred to the postgraduate clinic of the Department of Orthodontics, School of Dentistry, Oral Pathology and Maxillofacial Surgery at Leuven, were included in the study. The subjects and their parents were given a written explanation on the background of the study, its objectives and their involvement. After screening for their suitability and after good comprehension of the protocol, the parents all gave their written informed consent. The design of this study was approved by the Ethical Committee of the Catholic University of Leuven. The patients were selected if fulfilling the following inclusion criteria:

no smoking, absence of extensive dental restorations or adhesive fixed partial dentures, an orthodontic treatment plan without extractions, a sulcus bleeding index (Muhlemann & Son, 1971) of less than 0.3, no preexisting periodontal disease and no use of antibiotics during or up to 4 months prior to the study. The patients were asked whether they are right or left handed; in the right handed patients, the right quadrants are brushed more thoroughly, leading to a healthier gingival condition (Addy et al., 1987). All patients were right handed. Fourteen out of the 24 subjects were treated with headgear (headgear group) and received bands on the first upper molars for a period of 18 weeks prior to bonding brackets on the remaining upper teeth. In the headgear group it was possible to make intra-subject comparisons between the bonded and banded sites. The other 10 subjects were treated with brackets only (non-headgear group).

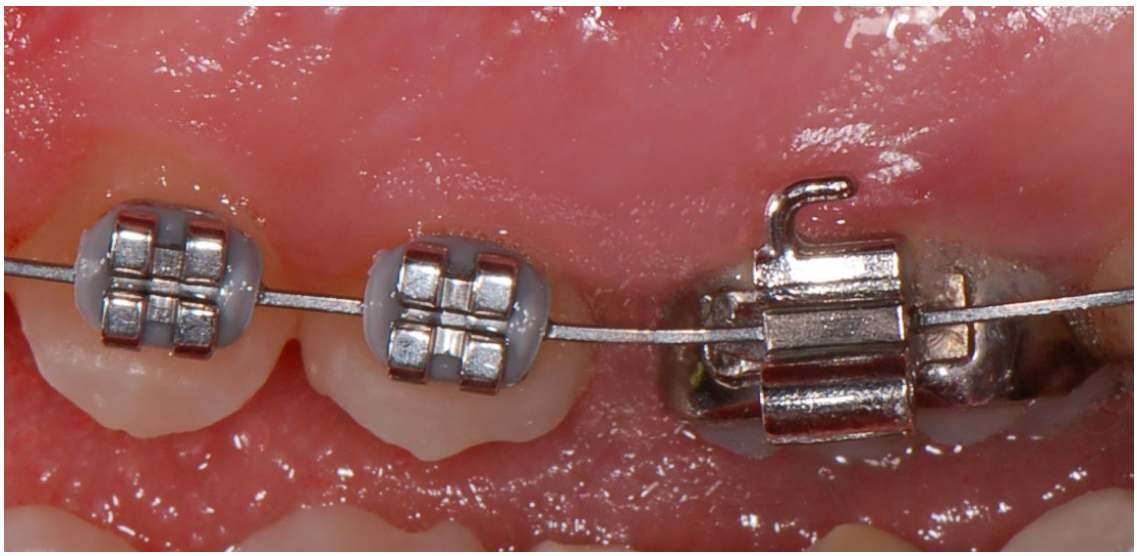

Figure IV.1: Clinical example of a subject in the headgear group. Image of the experimental setting at T18, after placement of the brackets on the remaining teeth and insertion of the orthodontic wire.

IV.2.b Experimental design

The study had a longitudinal, split-mouth design. Prior to the study all patients received a professional scaling and polishing followed by an oral hygiene instruction to ensure a healthy periodontal situation. During the study period the patients visited the clinic 4 or 5 times (Table IV.1). The first time (T0) to record the status praesens of the periodontium, to sample the sub and supragingival dental plaque and to place the molar bands. At the second visit after 18 weeks

(T18) the measurements and samples were repeated and brackets were bonded on the remaining upper teeth (headgear group). For the non headgear group, this was the first visit. The measurements and samples were repeated 2 (T20), 4 (T24) and 12 weeks (T36) later. Teeth 14 and 16 served as experimental sites whereas teeth 44 and 46 served as controls. The bonded sites were sampled every visit to follow the microbial and periodontal evolution, whereas the banded sites and the non-bonded lower jaw were only sampled at band placement (T0), bracket placement (T18) and after the eighteen weeks interval (T36) (Table IV.1). In the non-headgear group the samples (both microbial and crevicular) from teeth 16 and 14 were pooled, because both teeth were bonded. In the headgear group the samples from the bonded 14 and banded 16 were analyzed separately.

Table IV.1: Flow chart of the study. With the interventions depicted per contact (week x) and per site.

	contacts with the subjects (week) per site														
	0			18			20			24			36		
interventions	bonded	banded	control	bonded	banded	control	bonded	banded	control	bonded	banded	control	bonded	banded	control
molar-band placement		x													
bracket placement				x											
crevicular fluid	x	x	x	x	x	x	x			x			x	x	x
probing depth	x	x	x	x	x	x	x			x			x	x	x
bleeding on probing	x	x	x	x	x	x	x			x			x	x	x
supra-ging. microbiology	x	x	x	x	x	x	x			x			x	x	x
sub-ging. microbiology	x	x	x	x	x	x	x			x			x	x	x
oral hygiene instruction		x			x			x			x			x	
clinical photos		x			x			x			x			x	

A standardized oral hygiene instruction with an orthodontic toothbrush (Oral-B , Kirkland, P.Q. Canada) using the Bass technique and a single tufted brush (Oral-B , Kirkland, P.Q. Canada) was given. Interdental cleaning was instructed with extra fine interdental wood sticks (Oral-B , Kirkland, P.Q. Canada). The youngsters were able to use these wood sticks also after placement of the bands, the brackets and the wire. The patients were told to always brush for 3 minutes. The hygiene protocol was instructed using a model and afterwards the brushing of the subjects was analyzed and improved by the researcher (JvG) in order to achieve good comprehension (Ay et al., 2007). Every visit, the teeth were stained with erythrosine disclosing solution (4% erythrosine in water solution) in order to show the patients how to remove the remaining plaque.

IV.2.b.1 Band placement

At T. only the headgear group visited the clinic to place the molar bands. The teeth were pumiced with a rubber cup, the orthodontic bands were fitted and the correct size was selected. The gingival band margins were trimmed in order to be placed supragingival. After disinfecting the bands with alcohol and drying, the glasionomer cement (Unitek™ Multi-Cure Ionomer Orthodontic Band Cement, 3M Unitek, Monrovia, California) was mixed according to the manufacturers' instructions. The bands were then positioned firmly into place and any excess cement was removed from the occlusal and cervical margins of the bands. All band selection and cementation was performed by one operator (JvG). The cement was light cured (Dentsply QHL75 halogen curing light, Dentsply, Addlestone, Surrey, UK) for 60 seconds from the occlusal side. The preformed headgear was adjusted and the patients were instructed to wear it 14 hours a day. The standardized oral hygiene instruction was repeated.

IV.2.b.2 Bracket placement

At T18, all patients received brackets in the upper jaw. For the non-headgear group these were the first orthodontic attachments in the mouth. The headgear group did receive brackets on all remaining teeth in the upper jaw. The teeth were pumiced by means of a rubber cup and the quadrant to bond was isolated with cotton rolls and saliva suction. The one-step adhesive (Transbond™ Plus Self Etching Primer, 3M Unitek, Monrovia, California) was applied with a micro-

brush and the excess was blown away with dry air in the incisal/occlusal direction in order to avoid contact with the gingiva. The composite bonding material (Transbond™ Plus colour change adhesive, 3M Unitek, Monrovia, California) was applied to the bracket base, the bracket was pressed firmly onto the enamel surface and any excess of adhesive was removed. Then the composite was light cured (Dentsply QHL75 halogen curing light, Dentsply, Addlestone, Surrey, UK) for 30 seconds from the occlusal and for 30 seconds from the gingival direction. After placement of the brackets an initial nickel-titanium orthodontic wire was inserted and ligated to the brackets by means of elastic ligatures. The standardized oral hygiene instruction was repeated.

IV.2.b.3 Microbiology

The collection of the microbial samples is described in II.2.b.2.

IV.2.b.4 Crevicular Fluid

The collection and volume measurements of the crevicular fluid is described in II.2.b.3.

IV.2.b.5 Periodontal parameters

The periodontal scoring is described in II.2.b.4.

IV.2.b.6 Culture techniques

Analysis of the microbial samples is described in II.2.b.5.

IV.3 Statistics

The several time-treatment combinations were grouped into one factor. Differences between combinations were assessed using a linear mixed model with patient as random factor (as such the grouped character of the data was taken into account). The global level of significance (probability that the null hypothesis is falsely rejected, in any of the comparisons) was set up per parameter. Contrasts were set up to compare relevant treatments or outcomes on different days and a correction for simultaneous hypothesis testing was made according to Bretz (Bretz et al., 2001).

IV.4 Results

IV.4.a Microbiology (Table IV.2)

The supragingival CFU ratio (aerobe/anaerobe) on the banded sites did remain stable between week 0 and 18 (P=.57), but between week 18 and 36 a significant decrease was observed (P<.05). The bonded sites showed a faster shift: on week 24 the decrease already reached significance (P<.05) compared to week 18. The subgingival CFU ratio (aerobe/anaerobe) showed the same tendency (Table 2). On the banded sites, it did not decrease significantly between week 0 and 18 (P=.47), but between week 18 and 36 a significant decrease was observed (P<.05). For bonded sites the decrease in subgingival CFU ratio (aerobe/anaerobe) reached significance (P<.05) on week 20 compared to week 18 (somehow faster than the supragingival CFU ratio (aerobe/anaerobe)) and then stabilized. The banded sites showed a significantly higher CFU ratio (aerobe/anaerobe) for both the supra and subgingival plaque on week 18 compared to the bonded sites on week 36 (P<.05). The control sites showed no significant changes in subgingival and supragingival CFU ratio (aerobe/anaerobe) over time.

No significant differences concerning the CFU counts of black pigmented bacteria could be detected during this study.

IV.4.b Crevicular fluid

The crevicular fluid flow showed a significant increase over time for both the banded and the bonded sites, although there was a difference in pattern (Table IV.2). For the banded sites the flow initially did not change between week 0 and 18 (P=1.00), between week 18 and 36 a significant increase was seen (P<.05). The bonded sites showed a faster elevation of the crevicular fluid flow: already between week 18 and 20 it doubled (P<.05). Later the crevicular fluid production seemed to stabilize. The banded as well as the bonded sites showed significant elevated crevicular fluid flows on week 36 compared the control sites (P<.05). The banded sites showed a significantly lower crevicular fluid flow on T18 compared to the bonded sites on T36 (P<.05). The control sites did not show any significant change over time (Table IV.2).

Table IV.2: Supragingival and subgingival CFU ratio (aerobe/anaerobe), crevicular fluid flow, probing depth and the bleeding on probing tendency (bop). Displayed per site as mean (standard deviation) with the corresponding *P*-values of the comparisons between TX and T0 for the banded and control sites and between TX and T18 for the bonded sites (on T18 the brackets are placed).

	week	banded (n=14)		bonded (n=24)		control (n=24)	
		mean (SD)	P-value (TX/T0)	mean (SD)	P-value (TX/T18)	mean (SD)	P-value (TX/T0)
CFU ratio supragingival	0	0.57 (0.04)*	1	0.57 (0.02)*		0.57 (0.02)	1
	18	0.41 (0.02)	.57	0.56 (0.01)*	1	0.66 (0.01)	.99
	20			0.47 (0.01)	.85		
	24			0.35 (0.01)	<.05		
	36	0.21 (0.01)	<.05	0.24 (0.01)	<.05	0.52 (0.001)	1
CFU ratio subgingival	0	0.44 (0.02)*	1	0.49 (0.03)*		0.47 (0.02)	1
	18	0.27 (0.01)	.47	0.44 (0.01)*	1	0.43 (0.01)	1
	20			0.22 (0.01)	<.05		
	24			0.19 (0.01)	<.05		
	36	0.09 (0.01)	<.05	0.12 (0.004)	<.05	0.44 (0.01)	1
crevicular fluid flow (Periotron® read-out)	0	17.70 (0.66)*	1	16.40 (0.68)*		14.11 (0.67)	1
	18	19.49 (0.76)	1	13.85 (0.23)*	1	16.71 (0.28)	.97
	20			27.70 (0.68)	<.05		
	24			29.47 (0.44)	<.05		
	36	46.80 (2.44)	<.05	36.00 (0.64)	<.05	14.30 (0.30)	1
probing depth (mm)	0	1.85 (0.05)*	1	2.00 (0.04)*		2.04 (0.02)	1
	18	2.49 (0.03)	<.05	2.14 (0.01)*	1	2.23 (0.01)	.93
	20			2.49 (0.02)	<.05		
	24			2.62 (0.02)	<.05		
	36	2.98 (0.04)	<.05	2.94 (0.02)	<.05	2.28 (0.02)	.75
number of sites bop	0	0.34 (0.05)*	1	0.24 (0.04)*		0.12 (0.02)	1
	18	0.47 (0.06)	.992	0.21 (0.01)*	1	0.22 (0.09)	
	20			0.53 (0.03)	<.05		.66
	24			0.59 (0.02)	<.05		
	36	1.57 (0.04)	<.05	0.89 (0.03)	<.05	0.16 (0.01)	.94

* At this point in time the bands or brackets are not yet placed and the sites serve like a pseudo-control site.

A P-value of <.05 was considered significant.

IV.4.c Periodontology

The probing depth showed a significant increase over time for both the banded and the bonded sites (Table IV.2). For the banded sites a significant increase in probing depth was seen between week 0 and 18 (P<.05) and between week 18 and 36 (P<.05). The bonded sites showed significant increases in probing depth between week 18 and 20 (P<.05) and between week 20 and 36 (P<.05). At week 36 the probing depth was significantly higher for banded and bonded sites compared to control sites (P<.05). The banded sites showed no significant differences concerning the probing depths on T18 compared to the bonded sites on T36 (P=.078). The control sites showed no significant changes over time (Table IV.2).

The number of proximal sites bleeding on probing (bop) showed a significant increase over time for the banded as well as the bonded sites and again there was a difference in pattern (Table IV.2): for the banded sites the score initially did not change between week 0 and 18 (P=.992), between week 18 and 36 a significant increase was observed (P<.05). The bonded sites already showed a significant increase in bop two weeks after bracket placement (week 18 versus week 20) (P<.05). On week 36, the banded as well as the bonded sites showed significantly higher bop values than control sites (P<.05). The banded sites showed no significant differences concerning the number of sites bleeding on probing on T18 compared to the bonded sites on T36 (P=.17). The control sites showed no significant changes in the number of sites bleeding on probing over time (Table IV.2).

IV.5 Discussion

Although the sample size was relatively small, the split-mouth design enabled meaningful statistical results to be achieved with only 24 patients. Not all subjects were treated with bands on the upper first molars because some patients did not need a headgear and placement of brackets is then preferable as it is more accurate, which might lead to faster clinical results. The subjects should not be treated with the extraction of teeth because this might lead to increased probing depths at the sites proximal to the closed diastema.

Before the bands and brackets were placed the gingiva was healthy. This is important as several studies indicated an increased plaque accumulation in the presence of gingival inflammation (Daly & Highfield, 1996; Quirynen et al., 1991; Rowshani et al., 2004). Every visit the standardized oral hygiene instruction was repeated and improved if necessary. The compliance of patients to these oral hygiene measures was not assessed, but these differences between the patients are covered by the split-mouth design.

Some recent studies indicate that electric (ultrasonic) toothbrushes are more effective in reducing plaque and gingivitis in orthodontic patients (Costa et al., 2007). A meta-analysis on the other hand concluded that any inferences about clinical practice are precluded and greater standardization of the methodology in future trials is desirable to possibly draw conclusions (Kaklamanos & Kalfas, 2008). Subsequently manual toothbrushes were supplied to the subjects because it was difficult to provide electric toothbrushes to all of them.

It was not possible to measure the periodontal parameters in a blinded manner, but the researcher was blinded for previous scores. All laboratory analyses were performed with an unknown coding system, which was only revealed after the completion of the study.

The increased probing depths recorded during this study are most likely caused by pseudopocket formation or by deeper penetration of the probe into the weakened connective tissue (Anderson et al., 1991; Jansen et al., 1981). As these two processes could simultaneously contribute to the increase in probing depth, a distinction can not be made with the instruments used in this study. During the period of this experiment, gingivitis was induced but attachment loss probably did not occur (Gomes et al., 2007; Thomson, 2002).

The demonstrated increase in crevicular fluid is unlikely to be induced by the procedure of bracket placement. Phosphoric acid, a widely used etching product in both adhesive dentistry and orthodontics, can indeed create necrotizing effects on periodontal soft tissues (Forsberg, 1982). White ulcerative lesions were described in cases where the phosphoric acid was accidentally in contact with the mucosa for several minutes (Akman et al., 2005). To avoid these adverse effects in our study, a self etching primer was used. Dental adhesives can also have adverse effects on the gingiva: they are toxic to the

gingival fibroblasts *in vitro* (Huang et al., 2002). Particularly the residual monomers may cause gingival inflammation and irritation (Gioka et al., 2005). In order to avoid contact of the self-etching primer with the gingival margin, the product was only applied locally on a safe distance from the gingival margin with a little non soaked mini-sponge. The bonding material was directly polymerized and thus contact with the gingival margin could be prevented. Etching and bonding the control teeth was not considered because of this prevention protocol and the fact that we were interested in the effects of bracket placement, compared to non bonded (non-treated) teeth. The bands were attached with glass-ionomer cement and the brackets with composite material, leading to a possible difference in leakage of residual monomers. The materials used in this trial are commonly used in modern orthodontic treatments and these possible differences in monomer leakage are present in all patients and thereby should be considered.

Removal of the total amount of dental plaque each visit was impossible due to the orthodontic bands and brackets and the fact that trauma of the gingival margin would lead to increased gingival crevicular fluid flow. For these reasons, the absolute values of the colony forming units were not considered, and only the CFU ratio's (aerobe/anaerobe) were compared. This ratio between the aerobic and anaerobic bacteria is an important parameter to score the pathogenicity of the plaque (Petti & Renzini, 1994).

Bacterial culturing has been the classic diagnostic method widely used in the study of the composition of dental plaque and is still generally used as the gold or primary standard in periodontal research (Lau et al., 2004; Verner et al., 2006). The main advantages of this method are its capability to detect multiple bacterial species simultaneously, the possibility to obtain relative and absolute counts of the cultured species and it is the only method which is able to detect unexpected bacteria and to properly characterize new species and to permit the assessment of antibiotic sensitivity of the grown bacteria (Eick & Pfister, 2002; Perinetti et al., 2004). Disadvantages are that the anaerobic culturing procedure recovers only a part of the microscopic count obtained on the same plaque sample (Jervoe-Storm et al., 2005). This difference is usually attributed to the presence of uncultivable organisms, such as the various spirochetal species, which are not likely to be present in these youngsters. The culturing technique

relies on the detection of viable organisms and requires that samples are almost immediately processed upon acquisition in order to maximize bacterial survival, in conjunction with essential strict transport condition (Kamma et al., 2004). Furthermore the sensitivity of this method can be rather low: low numbers of a specific pathogen in a sample can stay undetected, but in this study we were interested in the over-all changes over time (Kamma et al., 2004).

GCF sampling by paper strips may significantly affect subgingival sampling at the same site but this influence will be similar at all sites, because the subgingival plaque sampling was always preceded by sampling the GCF (Mullally et al., 1994). Collection of the subgingival plaque before sampling the GCF was not considered because the traumatizing effect of the paper points would lead to increased GCF flows.

An over-all decrease in CFU ratio (aerobe/anaerobe) over time is seen in both the banded and the bonded sites. This increase in pathogenicity is accompanied by increased signs of gingival inflammation like the crevicular fluid flow and the bleeding on probing tendency (Table IV.2). The differences at the banded sites did not reach significance on T18 compared to T0 despite the fact that this is an 18 week period. The only periodontal parameter that increased significantly over this period, was the probing depth ($P<.05$). The minor impact of the bands was not expected but might be explained by their supragingival location and by the absence at that stage of an orthodontic archwire. From T0 to T18 the bands were combined with a headgear, this appliance can easily be removed to clean the banded sites. To our knowledge, no longitudinal reports to compare bands and brackets are available.

The bonded sites on the other hand showed a much faster reaction, not only for the microbial environment but also for the clinical periodontal parameters. After the first interval (T18-T20) all clinical periodontal parameters increased significantly, together with a significant decrease of the subgingival CFU ratio (aerobe/anaerobe). The reduction of the supragingival CFU ratio (aerobe/anaerobe) was slower and not significantly until T24, the decrease from T18 till T24 was significant ($P<.05$). This much faster reaction might be explained by the fact that the first wire is inserted at T18 directly after bracket placement, resulting in difficult approximate cleaning (Figure IV.1).

When a comparison is made between the banded sites on T18 and the bonded sites on T36, significantly lower CFU ratios (aerobe/anaerobe) were seen at bonded sites. In both cases the orthodontic attachments (bands and brackets) are in place for a period of 18 weeks. Here again the wire and the neighbouring brackets, resulting in decreased proximal cleaning, could explain the difference in response. A comparison between the banded and bonded sites on T36 did not show significant differences in sub- and supragingival CFU ratios (aerobe/anaerobe). Nevertheless a borderline significantly higher crevicular fluid flow (P=.068) and a significantly higher bleeding on probing tendency (P<.05) were seen at the banded sites compared to the bonded sites. This might be due to a longer impact of the biofilm on the banded sites.

The control sites did not show any significant changes over time, neither for the microbiological nor for the clinical periodontal parameters. This might be an indication that the significant changes after bracket placement reported in this study are a local event and that the placement of brackets does not significantly modify the microbiology and clinical periodontal parameters in the entire mouth.

No literature is available in which the microbial and clinical periodontal parameters are longitudinally monitored using a split-mouth design (brackets, bands and control). Recently Naranjo et al. (2006) conducted a trial in which they compared the subgingival dental plaque before the placement of brackets and after three months: scores for bleeding on probing, plaque index, and gingival index increased after bracket placement. *P. gingivalis, P. intermedia/P. nigrescens, T. forsythia,* and *Fusobacterium* species were significantly elevated in the experimental group after bracket placement compared to the control group without orthodontic therapy. So called super infecting microorganisms were also found (Naranjo et al., 2006). These results are in line with our findings.

Petti et al. (1997) performed a similar study with a duration of 6 months. They compared the influence on supra- and subgingival microbiota of fixed appliances with those of removable orthodontic appliances. Their data suggest that in well motivated patients keeping up with the oral hygiene, gingivitis and periodontitis did not occur during the first six months of treatment. The significant modification of microbiota shown in the subjects with fixed appliances

however suggests a risk for gingivitis in the following months of therapy. Moreover, a risk for periodontitis cannot be excluded (Petti et al., 1997). We found significant gingival consequences of orthodontic treatment with fixed appliances at an earlier stage. This may be due to explicit differences in methodology; in our study an intrasubject control was used (non-bonded teeth) and Petti et al. (1997) had no control group. Also we had a larger number of patients with fixed appliances (24 versus 15).

As discussed before, no studies in which banded and bonded sites are compared to controls are found by the authors. In an earlier study of our research group, van Gastel et al. (2007) conducted a randomized clinical trial with split-mouth design in which we compared the *de novo* plaque growth, the clinical periodontal parameters and the crevicular fluid flow between teeth bonded with two different bracket types and non-bonded control teeth. A significantly higher number of aerobe as well as anaerobe colony forming units (CFU) on the bonded teeth was found compared to the non-bonded controls. Also significant differences between the teeth bonded with the different bracket types were seen. This indicated that the design of orthodontic bracket systems can have a significant impact on the evolution of the surrounding microbial environment. The observed clinical periodontal parameters showed congruence with these changes in microbiology and significant differences between the two bracket types were proven for the gingival crevicular fluid flow and the periodontal pocket depth (Petti et al., 1997; van Gastel et al., 2007b). These results did raise our interest in comparing the effects of bands and brackets.

IV.6 Conclusion

From the data presented in this study we can conclude that the placement of both types of orthodontic attachments had a negative influence on both the microbial and the clinical periodontal parameters. Surprisingly, the placement of orthodontic bands alone did not have significant effects on the microbial and most of the clinical parameters (only the probing depths increased significantly), but after insertion of the orthodontic wire, changes did occur and the wire might be the trigger in the development of gingival inflammation. After the orthodontic attachments being in place for 18 weeks, the banded sites (on T18) did show a better microbial status and approximately the same clinical periodontal status compared to the bonded sites (on T36). Only the crevicular fluid differed significantly. Ultimately, the control sites did not show any significant changes over time, from which we may conclude that the alterations occurring around the bracket/band bearing teeth are strictly localized

The mere presence of a pathogenic microbial environment does not mean that the patient has periodontal disease, since the latter depends on a complex bacteria-host interaction that modulates the hosts individual response leading to inflammation and possibly, loss of attachment (Ramberg et al., 2003; Socransky et al., 1998; Socransky & Haffajee, 2005). However, if a slight inflammation is not controlled and plaque accumulation continues during the orthodontic treatment, this could have a detrimental impact on the patients' periodontium. For this reason, further research should be undertaken to elucidate the exact consequences of orthodontic treatment with fixed appliances during the complete treatment period.

CHAPTER V:

GCF FLOW AND COMPOSTION

CHANGES IN GCF FLOW AND COMPOSITION AFTER ONE YEAR OF ORTHODONTIC TREATMENT, A PREDICTIVE VALUE?

Aim: To monitor microbiological and periodontal changes after placement of orthodontic attachments over a 1 year period and to predict these changes by cytokine concentrations in the GCF. **Material and Methods:** This longitudinal split-mouth trial enrolled 24 patients. Microbiology, probing depth, bop and GCF flow and composition were assessed at baseline (Tb) and after one year (T52). A statistical comparison was made over time and between the banded and bonded sites. Prognostic factors for the clinical reaction on T52 in the GCF at Tb were determined. **Results:** Between Tb and T52, the pathogenicity of the plaque and all periodontal parameters increased significantly, but inter-site differences were not seen except for bop. The cytokine concentrations in the GCF did not differ significantly between the sites nor between Tb and T52. The IL-6 concentration in the GCF at Tb is a significant predictive value for the GCF flow on T52 ($P<.05$). The same relationship was found between the IL-8 concentration at Tb and the increase in probing depth at T52 ($P<.05$). **Conclusions:** IL-6 and IL-8 concentrations before orthodontic treatment showed to be significant predictive factors for some inflammatory parameters during treatment.

V.1 Introduction

The effect of orthodontic treatment on the composition of gingival crevicular fluid (GCF) is hardly explored. GCF reflects the immune and inflammatory reactions deriving from host-parasite interactions (Lamster, 1992; McCulloch, 1994) and bio-mechanical stress (Ren et al., 2007). It is a non-invasive method and until now a wide variety of substances involved in the inflammatory process and produced by the PDL cells in sufficient amounts to diffuse into the GCF, has already been studied (Giannopoulou et al., 2008). These authors compared the GCF composition of orthodontic patients to that of non-orthodontic controls. However, the wide inter-subject differences in GCF composition make reasonable comparisons difficult and warrant for prospective intra-subject studies.

Among many inflammatory and immune mediators identified in GCF, cytokines have attracted particular attention. Interleukin-6 (IL-6) is known to be a major modulator of inflammation in chronic local inflammatory reactions. It regulates the immune cell recruitment in the transition from the acute (recruitment of neutrophils) to the chronic (recruitment of monocytes) form of inflammation (Romano et al., 1997). Interleukin-8 (IL-8) is produced by a wide variety of cells (polymorphonuclear leukocytes, monocytes, macrophages, and fibroblasts) and plays a key role in the accumulation of leukocytes at the sites of inflammation (Bickel, 1993).

The aims of this study were to monitor the microbiological and clinical periodontal changes after placement of orthodontic attachments over a 1 year period and to link these changes to alterations in cytokine concentrations in the GCF. We hypothesized that the cytokine concentrations prior to orthodontic treatment have a predictive value for the clinical reaction during treatment.

V.2 Material and Methods

V.2.a Subjects

The subjects are described in IV.2.a.

Table V.1: General information on study population with data on, placement of bands, age and gender distribution.

			age (years)	
	n	headgear/ bands	mean	SD
male	10	6	14.6	0.7
female	14	8	14.7	1.4
total	24	14	14.6	1.1

V.2.b Experimental design

Prior to the study all patients received a professional scaling and polishing, followed by an oral hygiene instruction to ensure a healthy periodontal situation. The study had a longitudinal prospective design. The teeth 14 and 16 were sampled. In the non-headgear group these samples were pooled because both teeth were bonded. In the headgear group the samples were not pooled because the 16 was banded and the 14 bonded. The latter made it possible to compare the banded and bonded sites intra-subject. During the study period, the subjects were periodontally analyzed 2 or 3 times (Table V.2). The first time, minus 18 weeks (T-18), to record the status praesens of the periodontium, to sample the sub and supragingival plaque, and to install the molar bands. At the second visit after 18 weeks (T0) the measurements and samples were repeated and brackets were bonded on the remaining upper teeth (headgear group). For the non-headgear group, this was the first visit. At T0 also the initial orthodontic archwire was inserted. Thereby, T-18 is considered baseline for the banded sites and T0 is baseline for the bonded sites. After one year (T52) the final measurements were performed. A standardized oral hygiene instruction with an orthodontic toothbrush (Oral-B, Kirkland, P.Q. Canada) using the Bass technique and a single tufted brush (Oral-B, Kirkland, P.Q. Canada) was given. Interdental cleaning was instructed with extra fine interdental wood sticks (Oral-B, Kirkland, P.Q. Canada). The youngsters were able to use these wood sticks also after placement of the bands, brackets and the orthodontic wire. The patients were told to always brush their teeth for 3 minutes. The hygiene protocol was instructed using a model and afterwards the brushing of the subjects was analyzed and improved by a clinician (JvG) in order to achieve good comprehension (Ay et al., 2007).

Table V.2: Flow chart of the study. With the interventions depicted per contact (Tx) and per group. Tb is baseline meaning T-18 baseline for the headgear group, T0 baseline for the non-headgear group. T52 is the endpoint of the study.

	Tb				T52	
	T-18		T0			
interventions	headgear group	non-headgear group	headgear group	non-headgear group	headgear group	non-headgear group
molar-band placement	x					
bracket placement			x	x		
crevicular fluid sampling	x		x	x	x	x
probing depth measuring	x		x	x	x	x
bleeding on probing measuring	x		x	x	x	x
supra-gingival microbial sampling	x		x	x	x	x
sub-gingival microbial sampling	x		x	x	x	x
oral hygiene instruction	x		x	x	x	x
clinical photos	x		x	x	x	x

V.2.b.1 Band placement (T-18)

The procedure of band placement is described in IV.2.b.1.

V.2.b.2 Bracket placement (T0)

The procedure of band placement is described in IV.2.b.2.

V.2.b.3 Microbiology

The collection of the microbial samples is described in II.2.b.2.

V.2.b.4 Crevicular Fluid

The collection and volume measurements of the crevicular fluid is described in II.2.b.3. In order to measure the changes in GCF composition due to the increased plaque adhesion and the concomitantly provoked inflammatory reaction, the impact of tooth movement should be minimized. Before bracket placement this was not a problem, because no tooth movement was present at that moment. In order to minimize the effective tooth movement after one year of treatment we did not do orthodontic activations and the patients were not allowed to use intermaxillary elastics during a period of two months before the measurements were done.

After collecting all GCF samples, the fluid was extracted from the strips by adding 50 µl phosphate buffered saline (PBS) containing 0.05% Tween 20 and placing the vials at 4° C for 12 hours. After centrifugating the vials, the fluid was collected and again 50 µl PBS containing 0.05% Tween 20 was added and the whole procedure was repeated. The fluid distracted from the strips was pooled and stored at -70° C until analysis.

A Bio-Plex human cytokine assay for simultaneous quantification of Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), Interferon- γ (IFN-γ), Tumor Necrosis Factor- α (TNF-α), Monocyte Chemotactic Protein-1 (MCP-1) and Interferon-inducible Protein-10 (IP-10) was run according to the procedure recommended by the producer (Bio-Rad Laboratories, Hercules, CA, USA). For each cytokine, a standard curve (1/4 dilutions) ranging from 0.5 pg/ml (IL-4), 2 pg/ml (IL-2, IL-6, IL-8/CXCL8, IL-1. GM-CSF, IFN- γ, MCP-1/CCL2 and IP-10/CXCL10) or 8 pg/ml (TNF- α) to 32000 pg/ml was prepared. The filter plate was pre-wet with Bio-Plex assay buffer (Bio-Rad Laboratories, Hercules, CA, USA) and 50 µl of beads coupled to specific monoclonal antibodies against the analyzed cytokines were added/well. Subsequently, the standards and samples (12.5 µl/sample) were prepared using the Bio-Plex serum diluent kit (Bio-Rad Laboratories, Hercules, CA, USA) and 50 µl/tube was transferred to the test plate, followed by incubation (30 minutes) at room temperature on a shaker (300 rpm). After three washes with Bio-Plex wash buffer (Bio-Rad Laboratories, Hercules, CA, USA), detection antibody was added and the plate was again incubated (30 minutes, room temperature, 300 rpm) to allow interaction with the beads. After removal of excess detection antibody (three washes with Bio-Plex wash buffer), a final incubation with streptavidin-PE was performed (10 minutes, room temperature, 300 rpm). The beads were washed three times and re-suspended in 125 µl of Bio-Plex assay buffer before analysis in the Bio-Plex suspension array system. The data were analyzed using the Bio-Plex Manager software (v 4.1) with 5-parameter logistic regression curves.

V.2.b.5 Periodontology

The periodontal scoring is described in II.2.b.4.

V.2.b.6 Culture techniques

Analysis of the microbial samples is described in II.2.b.5.

V.3 Statistics

In order to compare the different groups, a linear mixed model was fit to the data with time and treatment as fixed factors. The data on baseline (Tb) were compared to those on T52 (week 52) [for the banded sites Tb is T-18 and for the bonded sites Tb is T0]. The repeated measurement aspect of the data was modelled by taking the patient factor as random variable. Residual analysis indicated that CFU counts, crevicular fluid flow and cytokine concentrations needed a log-transformation before analysis. Multiple comparisons between treatments and time groups were corrected for simultaneous hypotheses following the general linear hypothesis model, making a global confidence of 95% for all comparisons per variable. For variables with values below quantification limits the values below the quantification limit were replaced by a non-informative, randomly generated and uniformly distributed value between 0 and the quantification limit. As such, those variables could be modelled as a continuous variable. This process was repeated for 1000 times and a conservative evaluation of the P-values was made by only considering the variables for which 95% of the simulated P-values were below .05 as significant.

In order to find relations between the cytokine concentrations at baseline and the severity of inflammatory reaction after one year of treatment, a stepwise regression was performed with the periodontal parameter after one year orthodontic treatment as response and cytokine concentrations (IL-6, IL-8, IFN-γ, IP-10) at start as explanatory variables. Values entered in the model in the forward step and were omitted from the model in the backward step using a threshold P-value of 0.2. The variable modelling the difference in the models was by default used as the first variable in the model. Only variables having a P-value below .05 were considered as significant. A correlation coefficient would be difficult to interpret for the relations between the cytokine concentrations at baseline and the severity of inflammatory reaction after one year of treatment because the data are not bivariately normally distributed and

not independent (Sonnergaard, 2006). Instead we used the P-values of the regression reflect the strength of the relation.

V.4 Results (Table V.3)

V.4.a Microbiology

At Tb, no differences in CFU ratios (aerobe/anaerobe) of the dental plaque were present between the bonded and banded sites. No differences in CFU ratios (aerobe/anaerobe) between the two sexes on Tb were seen either. The supragingival and subgingival CFU ratios (aerobe/anaerobe) decreased significantly over time for both the banded and the bonded sites ($P<.05$). The decrease in ratio was not significantly different for the banded compared to the bonded sites. The prevalence of P*. gingivalis* increased over time for both the banded as well as the bonded sites, but intersite differences were not seen.

V.4.b Crevicular Fluid

Only for IL-6, IL-8, IFN-γ, IP-10 and MCP-1 the bioplex assay did provide enough readings above the detection limit in order to apply statistical analysis. No significant alterations in the cytokine concentrations occurred over time. Neither was there a significant difference in cytokine concentrations between the different sites at Tb and T52. When the absolute cytokine values per strip (and thereby per 30 seconds) were statistically analyzed, a two fold increase for IL-8 was seen between Tb and T52 ($P<.05$). Significant differences between the materials were not seen. The females showed a 20-fold higher concentration of IP-10 compared to the males at baseline ($P<.05$).

No relation between cytokine concentration and periodontal parameters was found at start. The concentrations of some cytokines at Tb however, seemed to be significant prognostic factors for the inflammation at T52 (Figure V.1). More specifically, the concentration of IL-6 at Tb had a significant predictive value for the GCF flow on T52 ($P<.05$). The same relationship was found between the IL-8 concentration at Tb and the increase in probing depth at T52 ($P<.05$). The higher the cytokine concentrations at Tb, the higher the probability for increased measurements at T52 for the GCF flow and probing depth.

Table V.3: The different microbial, periodontal and immunological parameters depicted per contact as mean (m) and standard deviation (SD). In the upper part of the table for the banded sites, on Tb and T52, combined with the P-value. In the lower part the same data is provided for the bonded sites.

		CFU ratio aerobe/anaerobe supragingival		CFU ratio aerobe/anaerobe subgingival		GCF flow (ul/30sec)		pocket depth (mm)		bleeding on probing (#approximal sites)		concentration IL-6 (pg/ul)		concentration IL-8 (pg/ul)		concentration IFN-γ (pg/ul)		concentration IP-10 (pg/ul)		concentration MCP-1 (pg/ul)	
		m	SD	m	SD	m	SD	m	SD	m	SD	m	SD	m	SD	m	SD	m	SD	m	SD
banded	Tb	0.61	.027	0.43	0.014	0.11	0.0021	2.17	0.55	0.27	0.44	9.89	0.69	2142	22.1	0.18	1.22	3.1	139	0.78	4.61
	T52	0.30	.009	0.11	0.009	0.26	0.0175	3.33	0.36	1.69*	0.48	0.64	0.31	1658	152	0.53	0.37	5.7	34.3	1.13	2.90
	P-value	<.001		.003		<.001		<.001		<.001		.68		.99		.29		.66		.99	
bonded	Tb	0.59	.009	0.47	0.009	0.08	0.0013	2.2	0.22	0.29	0.36	2,47	1.32	1655	66	0.24	0.18	38.4	35.2	1.85	1.01
	T52	0.30	.012	0.12	0.008	0.19	0.0029	3.15	0.46	1.15*	0.51	0.91	0.73	1766	110	0.73	0.16	38.1	41.2	1.56	1.48
	P-value	<.001		<.001		<.001		<.001		<.001		.55		.76		.75		.97		.98	

* There is a significant difference on T52 between the bonded and banded sites.

V.4.c Periodontology

None of the periodontal parameters differed significantly between the bonded and banded sites or between the sexes at Tb. The GCF flow, probing pocket depth and number of sites bleeding on probing increased significantly over time for both the bonded as well as the banded sites. Only for the bop measurements a significant difference (P<.05) in increase was seen between the banded and bonded sites. The number of approximal sites bleeding after probing was almost twice as high in the banded compared to the bonded sites. For the bop increase over time, there was also a significant time-sex-interaction (P<.05): the females (n=14) showed a significant difference (P<.05) in bop increase between the banded and bonded sites over this one year period whereas the males did not (P=.32).

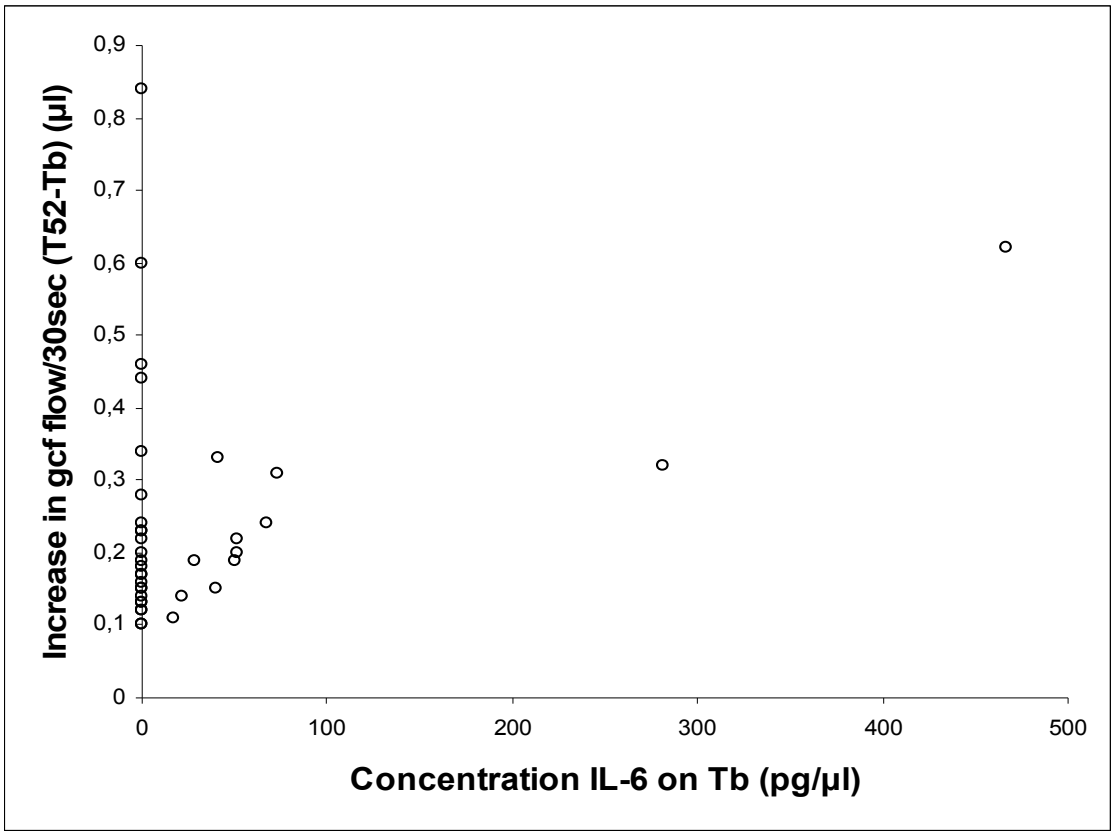

Figure V.1.A: Relation between the IL-6 concentration on Tb and the increase in GCF flow (T52-Tb).

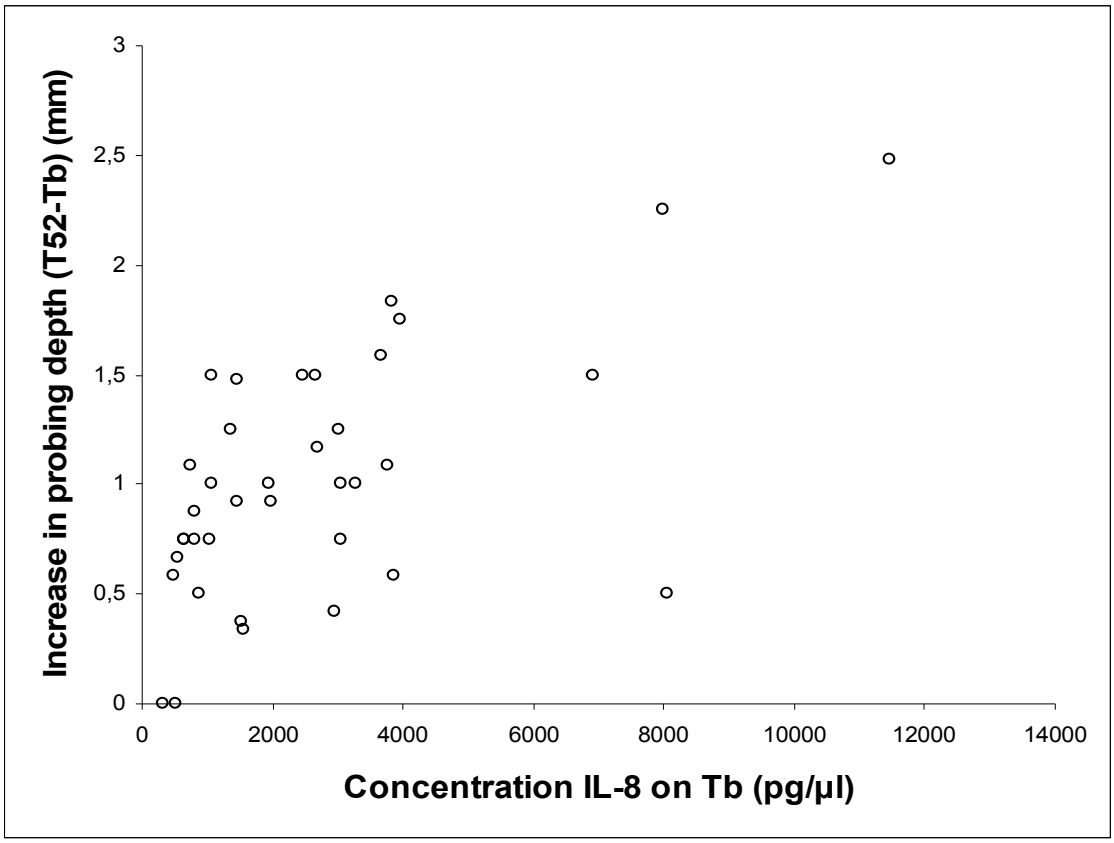

Figure V.1.B: Relation between the IL-8 concentration on Tb and the increase in ppd (T52-Tb).

V.5 Discussion

Although the sample size was relatively small, the split-mouth design enabled meaningful statistical results to be achieved with 24 patients only. Intra-subject rather than inter-subject comparisons were performed as more reliable results could then be expected. This is especially the case as it is known that the intra-subject differences in cytokine concentrations are rather high (Chipeta et al., 1998; Kowalczyk et al., 2002). The subjects are around the age of 14.5 years and at that age there are major differences between the subjects, especially between the males and females. Females are known to reach puberty earlier than the males with hormonal differences as a result. It seems logical that these differences do have an impact on the host response. Separate statistical analyses were thereby done for the two sexes.

Not all subjects were treated with bands on the upper first molars because some patients did not need treatment with a headgear. The subjects

should not be treated with the extraction of teeth because this might interfere with probing pocket depths at the sites proximal to the closed diastema.

Before the bands and brackets were placed, the gingiva was healthy. This is important as several studies indicated an increased plaque accumulation in the presence of gingival inflammation (Daly & Highfield, 1996; Quirynen et al., 1991; Rowshani et al., 2004). Every visit the standardized oral hygiene instruction was repeated and the oral hygiene was improved if necessary. The compliance of patients to these oral hygiene measures was not assessed, but the effects of potential differences between patients are covered by the split-mouth design.

Although it was not possible to measure the periodontal parameters in a blinded manner, the researcher was blinded for the previous scores. All laboratory analyses were performed using an unknown coding system, which was only revealed after the completion of the study.

The microbiological changes over time confirmed the results of a former study of our research group (van Gastel et al., 2008). During the short term follow up (18 weeks after bracket bonding) of that study significant elevations in microbial and clinical periodontal parameters were seen. Other authors showed comparable changes over time (Naranjo et al., 2006; Ristic et al., 2007). Petti et al. (1997) however did see a tendency towards the development of gingivitis but significant changes did not occur. A microbial comparison between bands and brackets was by our knowledge not made by any other author. Alexander (1991) however did find that the plaque index, the gingival index and pocket depths at banded molars were significantly elevated compared to bonded molars. He assumed that these differences in clinical periodontal reaction were attributed to alterations in the composition of the bacterial biofilm without sampling it. In our present study we did not find any significant differences in microbiology between the bonded and banded sites. This is contrary to the general expectation of orthodontists, assuming that the microbial/periodontal impact of the placement of a band is more harmful than that of a bracket.

Different methods of GCF collection for biochemical analysis are described (Griffiths, 2003). In this study the Periopaper® strips were used because they are easy to use and non-traumatic. One of the disadvantages of GCF sampling by paper strips is that it may significantly affect subgingival

microbial sampling at the same site. However, this influence will be similar at all sites, because the subgingival plaque sampling was always preceded by sampling the GCF (Mullally et al., 1994). Collection of the subgingival plaque before sampling the GCF was not considered because the traumatizing effect of the paper points would lead to increased GCF flows.

GCF cytokines reflect the immune and inflammatory reactions deriving from host-parasite interactions as well as from biomechanical stress and it is not possible to make a distinction between these two processes. In order to minimize the effect of tooth movement, the wires were not activated until 2 months before the samples were taken. The wire was not removed and thereby relapse will not occur. We wanted to measure the changes in GCF composition due to the increased plaque adhesion and the concomitantly provoked inflammatory reaction. To our knowledge this has not been performed so far. Before bracket placement this was not a problem, because no tooth movement was present at that moment. In order to minimize the effective tooth movement after one year of treatment we did not do activations and the patients were not allowed to use intermaxillary elastics during a period of two months before the measurements were done. Removing the archwire for a period was not considered because this would certainly lead to relapse and thereby passive tooth movement. After one year of orthodontic treatment the patients (none of them were treated with extractions) were in there finishing stages meaning there were only moderate activations. Başaran et al. (2006) followed the GCF concentrations of IL-2, 6 and 8 during an orthodontic treatment that consisted of extraction of 14 and 24 (Başaran et al., 2006). They came to the conclusion that during leveling (low forces) as well as during retraction (high forces) of the canines IL-6 and IL-8 were not elevated 21 days after of activation (Başaran et al., 2006). These data made us believe that after the two month period of no activations the contribution of tooth movement to the total amount of IL-6 and IL-8 could be ignored.

The volumes of GCF collected from each site are in the range of 0.1-0.6 µl. Characterization of the cytokines by conventional Enzyme-Linked Immuno Sorbent Assay (ELISA) would give us the possibility of detecting maximum 3 different cytokines without diluting the samples too much. Recently Giannopoulou et al. (2008) reported on a study in which they compared a group

of 24 subjects wearing fixed orthodontic appliances with a representative control group (Giannopoulou et al., 2008). They concluded that tooth movement with fixed orthodontic appliances resulted in increased expression of IL-1β and IL-8.

In order to detect differences in cytokine concentration over time and between the bonded and banded sites, these sites were followed over a one year period. Intra-subject rather than inter-subject comparisons were made because this would yield us more reliable results. Especially because inter-subject differences in cytokine concentrations are rather high. Puberty might be another factor causing large differences between individuals (Chipeta et al., 1998; Kowalczyk et al., 2002). Nevertheless, no significant differences over time and between the different materials were seen in this study when the cytokine concentrations were considered. This might be due to the fact that increased inflammation would lead to increased GCF production and thereby a dilution of the cytokines. When the absolute IL values per strip (and thereby per 30 seconds) were statistically analyzed, a two-fold increase for IL-8 was seen between T0 and T52 ($P<.05$). Differences between the materials were not seen.

V.6. Conclusion

IL-6 and IL-8 concentrations before orthodontic treatment showed to be significant predictive factors for some inflammatory parameters during treatment. Increased values of IL-6 at baseline were correlated with increased GCF flows after one year of orthodontic therapy ($P<.05$). High levels of IL-8 at baseline are predictive for higher probing depths at T52 ($P<.05$). To our knowledge, this information is new, but more research in this area is needed in order to elucidate the predictive value of those biomarkers. They might give clinical orthodontist the possibility to do a screening of the patients before active treatment in order to decide whether or not it is indicated to start orthodontic treatment especially in patients who consider treatment for mild aesthetic deviations. Until now it is not clear whether or not these worsened periodontal and microbial parameters will normalize after termination of the orthodontic therapy. Prospective studies to elucidate this topic should be carried out. Especially when the changes are not completely reversible, screening for the susceptibility with biomarkers IL-6 and IL-8 could be a helpful tool.

CHAPTER VI:

CHANGES AFTER BRACKET REMOVAL

LONGITUDINAL CHANGES IN MICROBIOLOGY AND CLINICAL PERIODONTAL PARAMETERS AFTER REMOVAL OF FIXED ORTHODONTIC APPLIANCES

Introduction: although more patients are treated orthodontically, no longitudinal studies are present in which orthodontic patients are monitored microbiologically and clinically from bracket placement up to three months posttreatment. **Materials and methods:** 24 patients were enrolled in this study. Microbiology (sub- and supragingival), ppd, bop and GCF flow were assessed at baseline (T1), at bracket removal (T2) and 3 months posttreatment (T3). A statistical comparison was made over time and between the banded, bonded and control sites respectively. **Results:** sub- and supragingival CFU ratio aerobe/anaerobe decreased significantly (relatively more anaerobes) on T2 compared to T1. Between T2 and T3 no significant increase in CFU ratio was seen, resulting in a significantly lower CFU ratio on T3 compared to T1 for the subgingival plaque. The difference concerning the supragingival plaque between T3 and T1 was not significant. The ppd, bop and GCF flow showed a significant increase between T1 and T2. Between T2 and T3 these variables decreased significantly, but the values remained significantly higher than they were on T0 (except for bop values at the bonded sites (P=.0646)) **Conclusion:** placement of fixed orthodontic appliances has a significant impact on both the microbial and clinical periodontal parameters. They only partly normalize after 3 months.

VI.1 Introduction

The placement of orthodontic bands and brackets influences plaque growth and maturation (Gomes et al., 2007; Lee et al., 2005; van Gastel et al., 2008). Significant differences in biofilm formation and periodontal reaction between different bracket types and between bonded teeth compared to control teeth are reported (van Gastel et al., 2007b).

Most studies reporting on gingival changes after bracket placement, suggested only reversible periodontal changes (Gomes et al., 2007; Thomson, 2002). A report on first premolar extraction cases however reported significant attachment loss during the orthodontic treatment at the extraction sites (Janson et al., 2003). Until now it is not clear whether or not these worsened periodontal and microbial parameters will normalize after the end of the active orthodontic therapy. Only in a few retrospective studies, the periodontal situation of orthodontically treated patients and non-treated controls was compared (Gomes et al., 2007). Prospective studies are needed to elucidate this topic. The aim of his study thereby is to monitor the microbial and clinical periodontal changes after the placement of orthodontic bands and brackets and to see whether or not these parameters will normalize after the end of active orthodontic therapy (Figure VI.1).



Figure VI.1: Clinical example of a patient before (A), directly (B) and three months after (C) debonding.

VI.2 Material and Methods

VI.2.a Subjects

The subjects are described in IV.2.a.

Table VI.1: Flow chart of the study. With the interventions depicted per contact (Tx) and per group. T1 is baseline (T-18 for the headgear group, T0 for the non-headgear group). T2 is at the day of debonding. T3 is 3 months after debonding and the endpoint of the study.

	T1				T2		T3	
	T-18		T0					
interventions	headgear group	non-headgear group	headgear group	non-headgear group	headgear group	non-headgear group	headgear group	non-headgear group
molar-band placement	x							
bracket placement			x	x				
debonding					x	x		
crevicular fluid sampling	x			x	x	x	x	x
probing depth measuring	x			x	x	x	x	x
bleeding on probing measuring	x			x	x	x	x	x
supra-gingival microbial sampling	x			x	x	x	x	x
sub-gingival microbial sampling	x			x	x	x	x	x
oral hygiene instruction	x			x	x	x	x	x
scaling and polishing	x		x	x			x	x

VI.2.b Experimental design

The study had a longitudinal prospective, split-mouth design. Prior to the study all patients received a professional scaling and polishing followed by an oral hygiene instruction to ensure a healthy periodontal situation. Teeth 14 and 16 were sampled, for the headgear group tooth 16 is a banded site and tooth 14 a bonded site. For the non-headgear group both teeth are bonded sites. During the study period, the subjects were periodontally analyzed 3 or 4 times (Table VI.1). The first time (T1) to record the status praesens of the periodontium, to sample the sub and supragingival plaque, and to install the molar bands. At the second visit after 18 weeks (T0) the measurements and samples were repeated

and brackets were bonded on the remaining upper teeth (headgear group). For the non headgear group this was the first visit. At T0 also the initial orthodontic archwire was inserted. Thereby, T-18 is considered baseline (T1) for the banded sites whereas T0 is considered baseline (T1) for the bonded sites. Just before bracket removal (T2) and three months after removal (T3) the measurements were performed again. A standardized oral hygiene instruction with an orthodontic toothbrush (Oral-B , Kirkland, P.Q. Canada) using the Bass technique and a single-tufted brush (Oral-B , Kirkland, P.Q. Canada) was given. Interdental cleaning was instructed with extra fine interdental wood sticks (Oral-B, Kirkland, P.Q. Canada). The youngsters were able to use these wood sticks also after placement of the bands, brackets and the orthodontic wire. The patients were told to always brush their teeth for 3 minutes. The hygiene protocol was instructed using a model and afterwards the brushing of the subjects was analyzed and improved by a clinician (JvG) in order to achieve good comprehension (Ay et al., 2007). Each visit, the teeth were stained with erythrosine disclosing solution (4% erythrosine in water solution) to show the patients how to remove the remaining plaque.

VI.2.b.1 Band placement (T-18)

The procedure of band placement is described in IV.2.b.1.

VI.2.b.2 Bracket placement (T0)

The procedure of band placement is described in IV.2.b.2.

VI.2.b.3 Debonding (T2)

The brackets were removed after sampling the plaque and measuring the periodontal parameters. After debonding, all the adhesive was removed from the teeth with a carbide bur (Komet, H282 204 010). After drying, the teeth were inspected again for remaining adhesive. When the teeth were free of bonding material, they were cleaned with manual and sonic scalers and pumiced.

VI.2.b.4 Three months after debonding (T3)

Three months after T2 the subjects were recalled to the clinic to sample the plaque and assessing the periodontal parameters.

VI.2.b.5 Microbiology

The collection of the microbial samples is described in II.2.b.2.

VI.2.b.6 Crevicular Fluid

The collection and volume measurements of the crevicular fluid is described in II.2.b.3.

VI.2.b.7 Periodontology

The periodontal scoring is described in II.2.b.4.

VI.2.b.8 Culture techniques

Analysis of the microbial samples is described in II.2.b.5.

VI.3 Statistics

A linear mixed model was fit to the data with time and type and their interaction as fixed factors. Repeated measurements on patients were taken into account by modelling the patients as a random factor. Except for pocket depth and bleeding on probing, values were log-transformed before analysis. Multiple comparisons between types and times were set up and a comparison of times was also performed for two types of subgroups: pathological or non-pathological pocket depth on one hand and upper and lower half of the GCF flow at removal of the bands/brackets on the other hand. Corrections for simultaneous hypothesis testing were performed via simulation.

VI.4 Results

VI.4.a Microbiology (Figure VI.2)

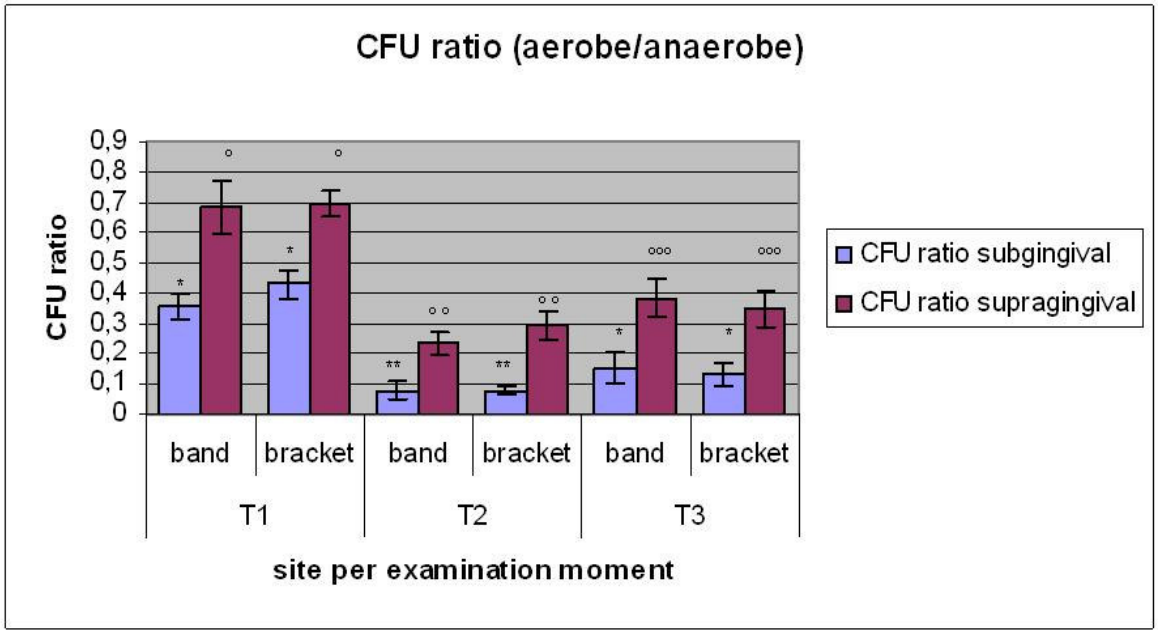

Figure VI.2: CFU ratio (aerobe/anaerobe) for the different banded and bonded sites per examination moment. Values are displayed as the mean and the standard deviation.
*/**/*** or °/°°significantly different (P<.05 was considered significant).

The banded and bonded sites showed the same tendency concerning the supragingival CFU ratio (aerobe/anaerobe): they decreased significantly between T1 and T2 (P<.05). Between T2 and T3 the supragingival CFU ratio (aerobe/anaerobe) increased, but not significantly. Ultimately the CFU ratio (aerobe/anaerobe) on T3 remained lower (factor 1.7 for the banded and 1.8 for the bonded sites) than on T1, but this difference is not significant. No significant differences between the banded and the bonded sites were seen at one of the contacts. The prevalence of *P. intermedia* in the supragingival dental plaque increased significantly from T1 to T2 for the banded sites (P<.05). The prevalence of *P. intermedia* dropped between T2 and T3 to normal values (T3 not significant different from T1). The subgingival CFU ratio (aerobe/anaerobe) also decreased significantly between T1 and T2 for both the banded and the bonded sites (P<.05). Between T2 and T3 the subgingival CFU ratio (aerobe/anaerobe) increased, but not significantly. Leading to significantly lower CFU ratios (aerobe/anaerobe) concerning the subgingival microbiology on T3 compared to T1 for both the bonded and the banded sites (P<.05). The

presence of *P. intermedia* in the subgingival plaque displayed no significant changes over time. When only the patients with the higher GCF flows on T2 were analyzed, a significant increase in subgingival CFU ratio (aerobe/anaerobe) was seen between T2 and T3 for both the banded and the bonded sites (P<.05). When a division was made between patients with probing depths higher and lower than 4 mm, no differences could be observed.

VI.4.b Crevicular fluid (Figure VI.3)

The banded as well as the bonded sites showed the same tendency: the GCF flow showed significant elevated levels at T2 compared to T1 (P<.05). Three months after debonding (T3), the GCF flow decreased significantly compared to T2, but remained significantly higher than the values on T1 (P<.05). No significant differences between the banded and the bonded sites were seen at one of the contacts.

VI.4.c Periodontology (Figure VI.4 and VI.5)

The probing depth showed a significant increase between T1 and T2 for both the banded as well as the bonded sites (P<.05). Between T2 and T3 the probing depth diminished significantly, but remained significantly higher than the probing depth on T1 (P<.05). When a division was made between patients with probing depths higher and lower than 4 mm, no differences could be observed. No significant differences between the banded and the bonded sites were seen at one of the contacts.

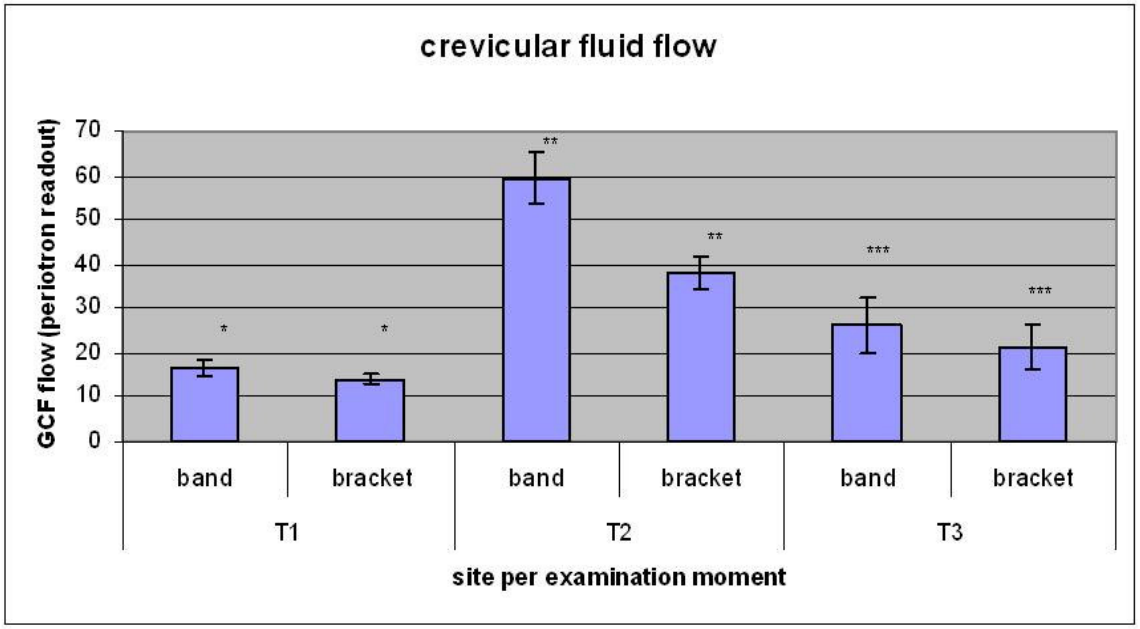

Figure VI.3: GCF flow (Periotron® readout) for the banded and bonded sites per examination moment. Values are displayed as the mean and the standard deviation.
*/**/*** are significantly different (P<.05 was considered significant).

The number of proximal sites that showed bleeding after probing (bop) increased significantly over time between T1 and T2 for both the banded and the bonded sites (P<.05). At T3 the banded sites still showed significantly higher number of sites bop than at T1 (P<.05). For the bonded sites the difference between T3 and T1 was borderline significant (P=.0646). When a division was made between patients with probing depths higher and lower than 4 mm, differences could be observed. The patients with probing depths lower than 4 mm on T2 did show a significant decrease in bop on T3 (P<.05). No significant differences between the banded and the bonded sites were seen at one of the contacts.

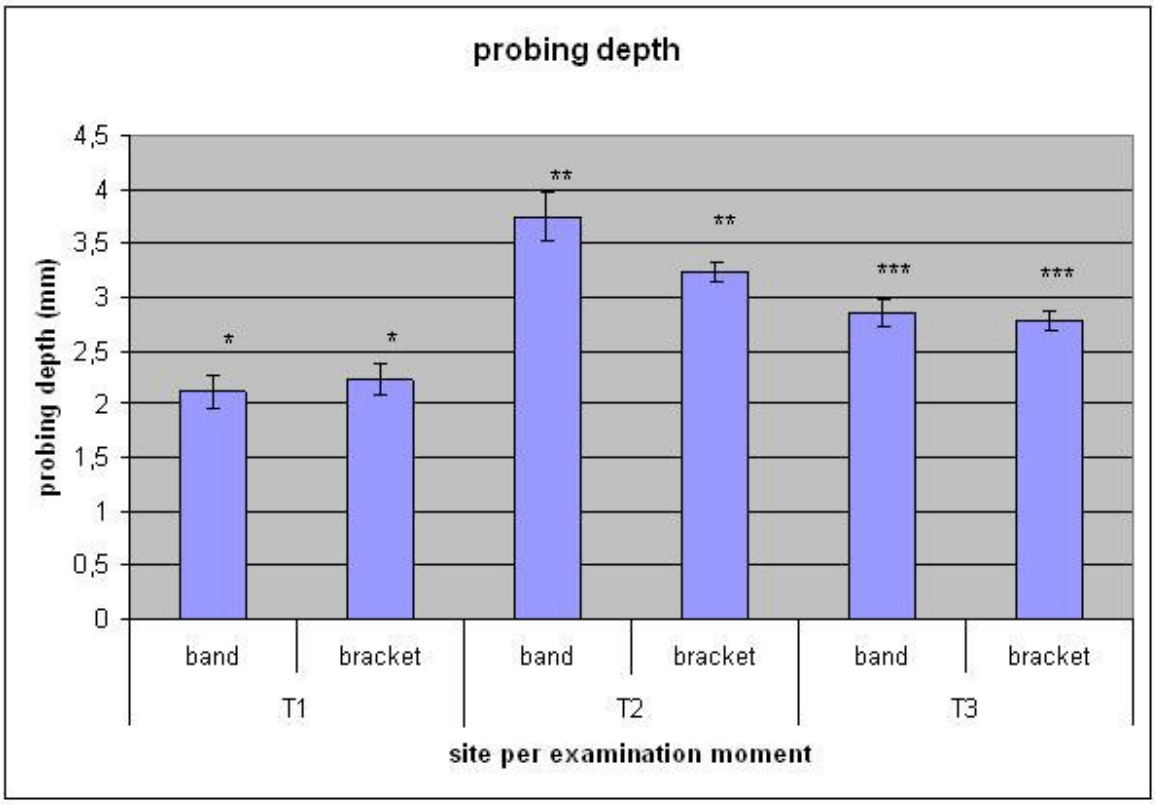

Figure VI.4: Probing depth (mm) for the banded and bonded sites per examination moment. Values are displayed as the mean and the standard deviation.
*/**/*** are significantly different (P<.05 was considered significant).

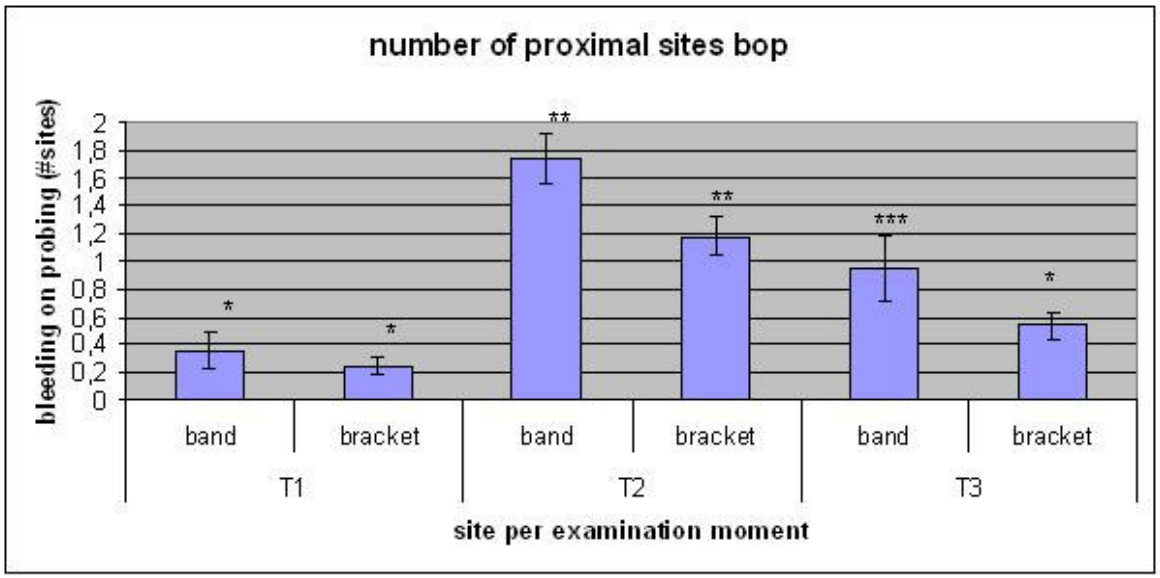

Figure VI.5: Number of sites bleeding on probing for the banded and bonded sites per examination moment. Values are displayed as the mean and the standard deviation.
*/**/*** are significantly different (P<.05 was considered significant).

VI. 5 Discussion

We carried out this prospective study because microbial and clinical periodontal data after completion of orthodontic treatment are largely lacking. The evaluation time was set at 3 months posttreatment because this was the time in our institute, at which a decision is made whether or not to perform a gingivoplasty in case of gingival hypertrophy after orthodontic treatment.

It was not possible to measure the periodontal parameters in a blinded manner, but the researcher was blinded for the previous scores. All laboratory analyses were performed with an unknown coding system, which was only revealed after the completion of the study.

Total removal of the dental plaque each visit was impossible due to the presence orthodontic bands and brackets; also this could give rise to a trauma of the gingival margin leading to increased gingival crevicular fluid flow. For these reasons, the absolute values of the colony forming units were not considered, and only the CFU ratios (aerobe/anaerobe) were compared. This ratio between the aerobic and anaerobic bacteria is an important parameter to score the pathogenicity of the plaque (Petti & Renzini, 1994).

GCF sampling by paper strips may significantly affect subgingival sampling at the same site but this influence will be similar at all sites, because the subgingival plaque sampling was always preceded by sampling the GCF (Mullally et al., 1994). Collection of the subgingival plaque before sampling the GCF was not considered because the traumatizing effect of the paper points would lead to increased GCF flows. A significant decrease in CFU ratio (aerobe/anaerobe) and thereby an increase in pathogenicity of the dental plaque was seen between the beginning and the end of treatment (Petti & Renzini, 1994). This alteration in microbial composition is described by others too (Kloehn & Pfeifer, 1974; Naranjo et al., 2006; van Gastel et al., 2008). The increased supragingival CFU ratio (aerobe/anaerobe) 3 months after debonding did not differ significantly anymore from the CFU ratio (aerobe/anaerobe) at T1. The subgingival CFU ratio (aerobe/anaerobe) at T3 on the other hand did differ significantly from the ratio at T1. This difference might be explained by the fact that the supragingival microbial composition is strongly influenced by the possibility of better oral hygiene after debonding.

The increased pathogenicity of the dental plaque and the concomitant periodontal changes during orthodontic treatment have already been described by several authors (Naranjo et al., 2006; Petti et al., 1997; van Gastel et al., 2008). Other studies did report on the microbial and periodontal changes such as plaque index, gingivitis index and probing depth after bracket removal (Sallum et al., 2004). The samples were taken twice: firstly during the final phase of orthodontic treatment and secondly 30 days after bracket removal and professional prophylaxis. Sallum et al. (2004) concluded that the periodontal signs of gingival inflammation decreased significantly after bracket removal. This improvement in periodontal health 30 days after bracket removal was accompanied by the reduction of the number of sites positive for *A. actinomycetemcomitans* and *B. forsythus.* The initial periodontal status before the start of treatment of the patients in the study performed by Sallum et al. was not taken into account and the conclusions drawn from this study are thereby not complete (Sallum et al., 2004).

In a study conducted by Gomes et al. (2007), the periodontal conditions of orthodontically treated and untreated individuals were compared (Gomes et al., 2007). Their results showed similar periodontal conditions for both groups and suggested an absence of permanent periodontal damage, traditionally related to fixed appliances, especially to banded molars. Their study population consisted of dental students with good oral hygiene, which makes it difficult to extrapolate these data to the general orthodontic population.

Recently, Thornberg et al. made a great attempt to document the changes of 8 putative periodontal pathogens in patients before, during and three months after fixed orthodontic appliance treatment (Thornberg et al., 2009). They concluded that the percentages of subjects with high pathogen counts increased significantly after 6 months of treatment compared to pretreatment. But the values returned to pretreatment level after 12 months of treatment. No pathogen level was significantly higher after 12 months of treatment and orthodontic treatment was found to be significantly protective for half of the periodontal pathogens (*E. corrodens, F. nucleatum, T. denticola and C. rectus*). These data are at odds with our microbial results.

VI.6 Conclusion

Placement of fixed orthodontic appliances has a significant impact on microbial and clinical periodontal parameters. All values worsened significantly at bracket removal (T2) compared to before bracket placement (T1). The supragingival CFU ratio (aerobe/anaerobe) normalized after 3 months, possibly because these sites are more sensitive to changes in oral hygiene. The subgingival CFU (aerobe/anaerobe) ratio three months after bracket removal (T3) remained significantly lower compared to before bracket placement (T1). Indicating that the changes induced by orthodontic treatment are partially irreversible. The periodontal values tended to normalize after debonding, but most periodontal values remained significantly elevated three months after bracket removal (T3) compared to before bracket placement (T1). It would be interesting to repeat these measurements after a longer period of time to elucidate the long term changes. If no further changes occur, it would be justified to perform a gingivoplasty 3 months after treatment to reduce the pocket probing depth and to make the sulcus healthy.

CHAPTER VII:

GENERAL DISCUSSION

GENERAL DISCUSSION

VII.1 Introduction

The recent years, the number of patients that undergo orthodontic treatment increased to approximately 50 percent of all adolescents (Kalsbeek et al., 2002). As stressed before, only a minority of these patients were treated because of dental or medical reasons. The impact of many orthodontic deviations on the periodontium has been used to justify orthodontic treatment, but only little evidence is present and the available studies often contradict each other (van Gastel et al., 2007a). Increased social well being can of course be a good reason for an orthodontic treatment too. Social response conditioned by the appearance of the teeth can severely affect an individuals attitude to life (Shaw et al., 1985). The (log term) periodontal consequences of these orthodontic interventions have not been elucidated.

Thereby the essential part of this PhD dissertation was investigating the microbial and clinical periodontal implications of an orthodontic treatment. During treatment with fixed orthodontic appliances we noted a significant alteration of both the sub and supragingival microbial environment and increased signs of gingival inflammation. To obtain the objectives, it was necessary to separate two effects:

1. changes due to the placement of orthodontic attachments (bands and/or brackets and the concomitant plaque adhesion,
2. the changes occurring due to tooth movement, because tooth movement is a type of tissue injury that leads to an inflammatory response (Tuncay, 2004).

In a clinical setting, during orthodontic treatment this distinction is difficult to make. The parameters of inflammation like increased GCF flow, ppd, bop can be caused by the increased plaque adhesion as well as by tooth movement.

VII.2 Influence of bracket design

The microbial and periodontal changes after bracket placement without orthodontic treatment were not studied *in vivo* before. As discussed in the introduction, it is difficult to distinguish the consequences of bracket placement (leading to increased plaque adhesion) from the effects of tooth movement. In order to make this distinction, in chapter II a *de novo* plaque growth experiment is described (van Gastel et al., 2007b). This study revealed that the bonded teeth showed a faster undisturbed plaque formation than the non-bonded control teeth, as could be expected. At the bonded sites, 2 till 8 times more CFU were counted (Table II.5 and II.6). The Speed® bonded teeth allowed significantly more plaque formation than GAC® bonded teeth. These results were consistent with most changes in crevicular fluid flow and the periodontal pocket depth measurements (Table II.3 and II.4). This indicates that the variation in bracket design has repercussions on the clinical reaction of the patient. There is not much information on this topic yet, but Türkkahraman et al. (2005) conducted a study to investigate the differences in microbial flora and periodontal status between two different archwire ligation techniques (Türkkahraman et al., 2005). These authors observed that although teeth ligated with elastomeric rings exhibited slightly higher numbers of microorganisms than teeth ligated with steel ligature wires, the differences were not statistically significant and can thus be ignored. Moreover the two archwire ligation techniques showed no significant differences concerning the gingival index, plaque index or pocket depths of the bonded teeth. However, teeth ligated with elastomeric rings were more prone to bleeding (Türkkahraman et al., 2005). Forsberg et al. (1991) conducted a split mouth study from which they concluded that teeth ligated with an elastomeric ring, exhibited a greater number of micro-organisms in the plaque than the incisor ligated with steel wire (Forsberg et al., 1991).

Anhoury et al. (2002) studied 32 metallic and 24 ceramic brackets from orthodontic patients at the day of debonding and showed no significant differences between metallic and ceramic brackets with respect to the caries-inducing *S. mutans* and *L. acidophilus* counts. Mean counts of 8 of 35 additional species differed significantly between metallic and ceramic brackets, with no obvious pattern favouring one bracket type over the other (Anhoury et al., 2002).

Recently, Pandis et al. (2008) explored whether the use of self-ligating brackets is associated with better values for periodontal indices because of the lack of these elastomeric modules and concomitantly, reduced availability of retentive sites for microbial colonization and plaque accumulation. They followed the periodontal status of the mandibular anterior teeth of 100 orthodontic patients (50 with conventional and 50 with self-ligating brackets) over an 18 months period. They came to the conclusion that under the conditions as applied in their study, the self-ligating brackets did not have an advantage over conventional brackets with respect to the periodontal status of the mandibular anterior teeth (Pandis et al., 2008).

The results of Chapter II clearly showed significant microbiological differences between the two bracket types and these differences were confirmed by the gingival crevicular fluid flow rates and the periodontal pocket depths. Because of the relation between the microbiological profiles of the different bracket types and the clinical reaction, it became of interest to compare biofilm formation on different bracket types by means of an *in vitro* study.

Several pilot studies were performed in order to optimize the circumstances in which this *in vivo* study took place. One of the conclusions was that the presence of saliva in the medium was necessary in order to give adhesion of the oral bacteria to the bracket surfaces. Saliva of the patient donating the dental plaque was used in order to have an optimal interaction between the salivary components and the micro-organisms, which is recognized as the primary step in biofilm formation and associated diseases (Walker & Sedlacek, 2007). All brackets were placed in one recipient in order to ensure the same environmental circumstances for each bracket. Because of the random placement of the brackets, differences in shear forces due to the medium could not contribute to differences in microbial adhesion between the

bracket types. The experimental setting as used in this study is not a common way to grow anaerobe biofilms (Walker & Sedlacek, 2007). The oscillating incubator and the poly-urethane box do not provide an anaerobic environment and BHI is certainly not the first choice medium to grow anaerobes. We wanted to simulate the aerobic oral environment and this probably underestimates the amount of anaerobe species, but this will be true for all bracket types. The fact that we did find anaerobe species (*P. intermedia*) will most likely be due to the mutual protective environment between the different species in the multi-species biofilm. The same process makes it possible to find anaerobic species in supragingival dental plaque. We did not identify the different aerobic species present in the microbial samples although these are responsible for the initial biofilm formation. Especially in trials with such short duration, these species are of great importance. But in the *in vivo* RCT (Chapter II), we clearly saw increased signs of inflammation and an increased pathological microbial environment at the bonded sites after 3 days (van Gastel et al., 2007b). This study showed that the biofilm was already shifted to more anaerobe and more pathogen species and those are the ones we are mostly interested in.

In order to compare the brackets in a fair way, the non self-ligating brackets were ligated with an elastomeric ligature because this is most comparable to the clinical situation. The results of this study indicate a major difference between the different brackets concerning the adhesion of supragingival oral microbes. The bracket types presenting high microbial adhesion also showed lower CFU ratios (aerobe/anaerobe) and higher CFU *P. intermedia* counts, probably due to a thicker and more mature biofilm leading to a more anaerobe environment at the base of this biofilm. *P. intermedia* is known to be one of the species that are elevated in orthodontic patients compared to non-orthodontic controls (Naranjo et al., 2006).

In our study, both ceramic brackets (E and F) were in the high microbial adhesion group and displayed the lowest CFU ratios (aerobe/anaerobe), this is in congruence with the findings of some researchers (Ahn et al., 2007; Brusca et al., 2007). Others did not find significant differences between brackets manufactured from different materials (Anhoury et al., 2002). Eliades et al. studied the basic principles of bacterial adhesion (Eliades et al., 1995): they compared the wettability and the composition of salivary films on raw

orthodontic bracket materials by contact angle measurements. Stainless steel presented the highest critical surface tension and total work of adhesion, indicating an increased potential for micro-organisms to attach on metallic brackets. These findings are not in line with our data, as we saw significantly higher adhesion on the ceramic compared to the metal brackets. The brackets ligated with an elastomeric ring did not per se show higher bacterial counts compared to the self-ligating brackets. For the metallic brackets (A, D, E, F and G) we can even conclude that the self-ligating brackets showed significantly higher (P<.05) bacterial counts and a lower CFU ratio (aerobe/anaerobe) than the classic brackets with elastomeric rings in this in vitro study. The results from this in vitro study can not be extrapolated to an in vivo setting. Further comparisons by means of a RCT with several different bracket types could fill the lacuna in this field of clinical orthodontics.

From the in vivo comparison we can conclude that the difference in bracket design does have its microbial and clinical periodontal implications. This is important knowledge to the manufacturers of orthodontic materials. Until now only little scientific research is performed before bracket materials are released on the market. Because legal direction are and will be lacking, it should be the task of the orthodontists to demand proper scientific research in this area before brackets are used clinically.

VII.3 Microbial and periodontal changes during orthodontic treatment

Alexander (1991) already showed that plaque index, gingival index and pocket depths were significantly higher at banded sites compared to control sites, indicative of increased inflammatory activity where bands were cemented. Once the appliances were removed, both areas returned to pretreatment levels (Alexander, 1991). The placement of orthodontic bands and brackets might influence the biofilm formation and maturation. In general, orthodontists assume that placement of bands has a greater impact on the microbial environment than brackets, due to the bigger volume of this attachment. No studies in which bands and brackets were compared have been published however. Lee et al (2005) reported significantly higher prevalences of putative periodontal pathogens in subgingival plaque from gingivitis lesions in orthodontic patients

compared to non-orthodontic patients (Lee et al., 2005). Ristic et al. (2008) recently conducted a trial in which they followed 32 adolescents during the first 6 months of their fixed appliances treatment (Ristic et al., 2007). They reported that values of pocket probing depth, total number of microorganisms and number of patients with positive findings of *P. intermedia* and other periodontopathogenic anaerobes increased from the start of treatment to a maximum obtained after two months. Both clinical and microbiological values decreased 6 months after the beginning of orthodontic therapy.

These data are not in complete congruence with ours. We detected an over-all decrease in CFU ratio (aerobe/anaerobe) over time, both at the banded and bonded sites. This increase in pathogenicity is accompanied by increased signs of gingival inflammation like the crevicular fluid flow and the bleeding on probing tendency (Table IV.2). We did not observe a reduction in the pathogenicity of the dental plaque nor in the periodontal parameters after a period of 18 weeks of active treatment, as described by others (Ristic et al., 2007). In our study, the differences at the banded sites did not reach significance during the period that they were the only attachments in the mouth, despite the fact that this was an 18 week period. The only periodontal parameter that increased significantly over this period, was the probing depth ($P<.05$). The minor impact of the bands during this period was not expected but might be explained by their supragingival location and by the absence at that stage of an orthodontic archwire. During this period, the bands were combined with a headgear, this appliance can easily be removed to clean the banded sites. After placement of the brackets on the remaining teeth and insertion of the wire, significant changes did occur (T18-T36).

The bonded sites on the other hand showed a much faster reaction, not only concerning the microbial environment but also for the clinical periodontal parameters. Already two weeks after bracket (T20) placement all clinical periodontal parameters increased significantly, together with a significant decrease of the subgingival CFU ratio (aerobe/anaerobe). The reduction of the supragingival CFU ratio (aerobe/anaerobe) was slower and not significant until 6 weeks after bracket placement (T24). The much faster reaction at the banded sites might be explained by the fact that the first wire is inserted directly after bracket placement, resulting in difficult approximate cleaning.

The control sites did not show any significant changes over time, neither for the microbiological nor for the clinical periodontal parameters. This might be an indication that the significant changes after bracket placement reported in this study are a local event and that the placement of brackets does not significantly modify the microbiology and clinical periodontal parameters in the entire mouth.

No literature is available in which the microbial and clinical periodontal parameters are longitudinally monitored using a split-mouth design (brackets, bands and control). Naranjo et al. (2006) conducted a trial in which they compared the subgingival dental plaque before the placement of brackets and after three months. Scores for bleeding on probing, plaque index, and gingival index increased after bracket placement. *P. gingivalis, P. intermedia/P. nigrescens, T. forsythia,* and *Fusobacterium* species were significantly elevated in the experimental group after bracket placement compared to the control group without orthodontic therapy. So called superinfecting microorganisms were also found (Naranjo et al., 2006). These results are in line with our findings.

Petti et al. (1997) performed a similar study with duration of 6 months. They compared the influence on supra- and subgingival micro flora of fixed appliances with those of removable orthodontic appliances. Their data suggest that in well motivated patients keeping up with the oral hygiene, gingivitis and periodontitis did not occur during the first six months of treatment. The significant modification of oral microbiota shown in the subjects with fixed appliances however suggests a risk for gingivitis in the following months of therapy. Moreover, a risk for periodontitis cannot be excluded (Petti et al., 1997). We found significant gingival consequences of orthodontic treatment with fixed appliances at an earlier stage. This may be due to explicit differences in methodology: in our study an intrasubject control was used (non-bonded teeth) and Petti et al. (1997) had no control group. Also we had a larger number of patients with fixed appliances (24 versus 15).

In general we can conclude that the placement of bands does not have a more negative influence on both the microbial and the clinical periodontal parameters than the placement of brackets. The placement of the attachment without the orthodontic wire does not have significant effects on the

periodontium. Taking out the wire for a period during active orthodontic treatment in order to let the gingiva become healthy in case of gingivitis seems justified. The fact that the alterations occurring around the bracket/band bearing teeth are strictly localized, could be of interest in periodontal cases with pre-existing localized pathology. The alignment of the upper teeth in an adult patient with periodontal breakdown in the lower anterior region would not influence the microbial and periodontal situation around these lower anterior teeth.

VII.4 Immunological reaction in the GCF during orthodontic treatment.

The composition of the gingival crevicular fluid, and the changes during orthodontic treatment became of great interest last years. Especially the immunological reaction due to tooth movement has thoroughly been studied. GCF reflects the immune and inflammatory reactions deriving from host-parasite interactions (Lamster, 1992; McCulloch, 1994) and bio-mechanical stress (Ren et al., 2007). Analysis of the GCF is a non-invasive method and until now a wide variety of substances involved in the inflammatory process and produced by the PDL cells in sufficient amounts to diffuse into the GCF, has already been studied (Başaran et al., 2006; Giannopoulou et al., 2008; Toygar et al., 2008). IL-6 and IL-8 are both proinflammatory cytokines: they alarm cytokines, inducing vascular dilatation with increased permeability and enhancing inflammatory response. IL-6 regulates immune responses in inflammation sites and has an autocrine/ paracrine activity that stimulates osteoclast formation and bone resorbing activity of preformed osteoclasts (Kurihara et al., 1990; Okada et al., 1997). In the mononuclear phagocytic system, several stages are required for local tissue cells to turn into full functional multinucleated cells. IL-8 is a potent proinflammatory cytokine that has a key role in the recruitment and activation of neutrophils during inflammation and is secreted mainly by monocytes (Vandevska-Radunovic et al., 1997).

We wanted to measure the changes in GCF composition due to the increased plaque adhesion and the concomitantly provoked inflammatory reaction. To our knowledge this has not been performed so far. Before bracket placement this was not a problem, because no tooth movement was present at

that moment. In order to minimize the effective tooth movement after one year of treatment we did not do activations and the patients were not allowed to use intermaxillary elastics during a period of two months before the measurements were done. Removing the archwire for a period was not considered because this would certainly lead to relapse and thereby passive tooth movement. After one year of orthodontic treatment the patients (none of them were treated with extractions) were in their finishing stages meaning there were only moderate activations. Başaran et al. (2006) followed the GCF concentrations of IL-2, 6 and 8 during an orthodontic treatment that consisted of extraction of 14 and 24 (Başaran et al., 2006). They came to the conclusion that during levelling (low forces) as well as during retraction (high forces) of the canines, IL-6 and IL-8 were not elevated 21 days after of activation (Başaran et al., 2006). These data made us believe that after the two month period of no activations the contribution of tooth movement to the total amount of IL-6 and IL-8 could be ignored.

The results presented in Chapter V indicate that no significant changes in cytokine concentrations were present, nor over time neither between the banded and the bonded sites. When the absolute cytokine values per strip (and thereby per 30 seconds) were statistically analyzed, a two fold increase for IL-8 was seen between baseline and during treatment. Total amounts and concentrations were used, because very small errors in volume determination can lead to large errors in estimates of final concentrations if the total volume collected is small. IL-6 and IL-8 concentrations before orthodontic treatment showed to be significant predictive factors for some inflammatory parameters during treatment. Increased values of IL-6 at baseline were correlated with increased GCF flows after one year of orthodontic therapy. High levels of IL-8 at baseline are predictive for higher probing depths at T52. To our knowledge, this information is new and could contribute to the decision whether or not to start an orthodontic treatment in susceptible patients. More research in this area is needed in order to elucidate the predictive value of those biomarkers.

Recently Giannopoulou et al. (2008) reported on a study in which they compared a group of 24 subjects wearing fixed orthodontic appliances with a representative control group. The results demonstrate that young adults with fixed orthodontic appliances had significantly higher levels of IL-1b and IL-8

compared to children and adolescents without orthodontic treatment. The levels of IL-4 did not differ between the two groups (Giannopoulou et al., 2008). These differences between the groups were associated with the presence of bleeding on probing and the presence of inflammatory gingival overgrowth. These authors stated that there was no difference in oral hygiene between the two groups and thereby the difference in cytokine concentration was due to tooth movement. Surprisingly, the oral hygiene was assessed using the presence or absence of plaque and not by quantification or by qualification of the biofilm.

The results show that IL-6 and IL-8 concentrations before orthodontic treatment showed to be significant predictive factors for some inflammatory parameters during treatment. This new information might seem academically, but in the future it might give clinical orthodontist the possibility to do a screening of the patients before active treatment. This to decide whether or not it is indicated to start orthodontic treatment especially in patients who consider treatment for mild aesthetic deviations.

VII. 5 Microbial and periodontal changes after debonding

Most studies agree that during orthodontic treatment a microbial shift occurs towards more pathogen species. This induces increased pocket depths, most likely due to pseudo pocket formation and/or by deeper penetration of the probe into the weakened connective tissue (Anderson et al., 1991; Jansen et al., 1981). By penetration into the epithelium the measurement is higher than the actual pocket depth. As these two processes could simultaneously contribute to the increase in probing depth, a distinction can not be made with the instruments used in these studies. During the period of this experiment, gingivitis was induced but attachment loss probably did not occur (Gomes et al., 2007; Thomson, 2002). Some however, reported a significant loss of attachment during the orthodontic treatment (Janson et al., 2003). Until now it is not clear whether or not these worsened periodontal en microbial parameters will normalize after termination of the orthodontic therapy. Only in a few retrospective studies, the periodontal situation of orthodontically treated patient and non-treated controls was compared (Gomes et al., 2007). Their results showed similar periodontal conditions for both groups and suggested an

absence of permanent periodontal damage, traditionally related to fixed appliances, especially to banded molars (Gomes et al., 2007). Their study population consisted of dental students with good oral hygiene, which makes it difficult to extrapolate these data to the general orthodontic population. Therefore the prospective study given in Chapter VI was set up to monitor the microbial a clinical periodontal changes after the placement of orthodontic bands and brackets and to see whether or not these parameters will normalize after termination of the orthodontic therapy.

The results from Chapter VI showed that placement of fixed orthodontic appliances has a significant impact on microbial and clinical periodontal parameters. All values worsened significantly at bracket removal (T2) compared to the initial situation before bracket placement (T1). The supragingival CFU ratio (aerobe/anaerobe) normalized after 3 months, possibly because these sites are more sensitive to changes in oral hygiene. The subgingival CFU (aerobe/anaerobe) ratio three months after bracket removal (T3) remained significantly lower compared to before bracket placement (T1), indicating that the changes induced by orthodontic treatment are partially irreversible. The periodontal values tended to normalize after debonding, but most periodontal values remained significantly elevated three months after bracket removal (T3) compared to before bracket placement (T1).

Recently, Thornberg et al. (2009) made a great attempt to document the changes of 8 putative periodontal pathogens in patients before, during and three months after fixed orthodontic appliance treatment. They concluded that the percentages of subjects with high pathogen counts increased significantly after 6 months of treatment compared to pretreatment. But the values returned to pretreatment level after 12 months of treatment. No pathogen level was significantly higher after 12 months of treatment and orthodontic treatment was found to be significantly protective for half of the periodontal pathogens (*E. corrodens, F. nucleatum, T. denticola and C. rectus*) (Thornberg et al., 2009).

In conclusion, we can state that during orthodontic treatment with fixed orthodontic appliances all microbial and clinical periodontal parameters worsened significantly. During the three months posttreatment follow-up period the supragingival CFU ratio (aerobe/anaerobe) normalized, but the subgingival CFU (aerobe/anaerobe) ratio remained significantly lower. Most periodontal

values remained significantly elevated three months after bracket removal. The fact that the periodontium was still inflamed due to the low subgingival CFU ratio (aerobe/anaerobe), probably caused by a remained pseudo pocket, justifies a gingivoplasty three months after bracket removal.

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CURRICULUM VITAE

Jan van Gastel was born on May 18th 1980 in Sint-Oedenrode, The Netherlands. He completed secondary school in 1998 at the Elde College, Schijndel, The Netherlands. In 2003 he graduated as DDS at the Catholic University Leuven.
In 2003 he started his PhD training under guidance of Prof. dr C. Carels and Prof. dr. M. Quirynen. In 2005 his postgraduate training orthodontics and master after master program was started.

He is member of the Belgische Beroepsvereniging van Nederlandstalige Orthodontisten (BBNO), the Vereniging van Orthodontisten (VvO) the European Orthodontic Society (EOS) and the World Federation of Orthodontists (WFO).

1. COMMUNICATIONS AT INTERNATIONAL CONFERENCES

(only available as abstract)

Van Gastel Jan, Quirynen Marc, Carels Carine. *The influence of bracket design, on microbial and periodontal parameters in volunteering dental students with good oral hygiene, a split-mouth design.* Oral presentation. 79ème réunion scientifique de la Société Française d'Orthopédie Dento Faciale. Brussels, Belgium. December 2005

Van Gastel Jan, Quirynen Marc, Coucke Wim, Carels Carine. 2006. *The influence of bracket design, on microbial and periodontal parameters in vivo.* Poster presentation. European Orthodontic Society congress. Vienna, Austria. June 2006

Van Gastel Jan, Quirynen Marc, Teughels Wim, Coucke Wim, Carels Carine. 2008. *Microbial and clinical periodontal changes in microbial and periodontal after bracket placement*. Poster presentation. Orthodontic Society congress. Lisbon, Portugal. June 2008

Van Gastel Jan, Quirynen Marc, Teughels Wim, Vandamme Jo, Carels Carine. Longitudinal changes in gingival crevicular fluid, flow and composition during orthodontic treatment with fixed orthodontic appliances. Poster presentation. European Orthodontic Society congress. Helsinki, Finland. June 2009

2.PUBLICATIONS

Van Gastel Jan, Carels Carine. Onderzoek van creviculaire vloeistof (Chapter in a book). Tandheelkundig jaar 2006; 82-96; Bohn Stafleu van Loghum

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