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Longitudinal monitoring of subgingival colonization by *Actinobacillus actinomycetemcomitans*, and crevicular alkaline phosphatase and aspartate aminotransferase activities around orthodontically treated teeth

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Abstract

Objectives: During orthodontic treatment, changes in subgingival plaque colonization and tissue inflammation and remodelling have been described. This study uses a longitudinal design to examine subgingival colonization of *Actinobacillus actinomycetemcomitans* (*Aa*) and alkaline phosphatase (ALP) and aspartate aminotransferase (AST) activities in gingival crevicular fluid (GCF) in order to assess whether these parameters have potential as biomarkers of tissue responses to orthodontic tooth movement in humans.

Materials & Methods: Twenty-one patients (ages: 11.2–22.5; mean 17.1 ± 3.3 years) participated in the study. An upper canine from each patient undergoing treatment for distal movement served as the test tooth (DC), and its contralateral (CC) and antagonist (AC) canines were used as controls. The CC was included in the orthodontic appliance, but was not subjected to the orthodontic force; the AC was free from any orthodontic appliance. The subgingival plaque and GCF around the experimental teeth was harvested from both mesial and distal tooth sites immediately before appliance activation and on day 28. Clinical gingival condition was evaluated at the baseline and at the end of the experimental period. *Aa* colonization was determined by culture methods, while ALP and AST activities were evaluated spectrophotometrically.

Results: Throughout the study, the clinical conditions worsened in both the DCs and the CCs as compared with the baseline, whereas no significant differences were found between the DCs and the CCs, or between mesial and distal sites of each of these teeth on day 28. In the ACs, clinical parameters remained at baseline levels throughout the study. Similar results were found for *Aa* colonization, which increased significantly on day 28 in the DC and CC groups. On day 28, ALP and AST activities were

significantly elevated in all sites from the DC and CC groups as compared with the ACs, where, conversely, enzymatic activities remained at the baseline levels. However, ALP activity in the DC group was significantly greater than in the CCs at mesial (tension) sites on day 28, while AST activity in the DCs was significantly elevated as compared with the CC group at the distal (compression) sites. Greater ALP activity in the DC group was observed at the tension sites compared with the compression sites on day 28.

Conclusions: Our results suggest that *Aa* subgingival colonization, and ALP and AST activities in GCF reflect the tissue responses that occur in the periodontium during orthodontic treatment.

Key words: *Actinobacillus actinomycetemcomitans*; alkaline phosphatase; aspartate aminotransferase; gingival crevicular fluid; tooth movement

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Several studies have reported inflammation of gingival tissues during fixed orthodontic therapy (Zachrisson & Zachrisson 1972; Trossello & Gianelly 1979). This condition has been related to less effective oral hygiene measures due to the presence of fixed orthodontic appliances, which can result in an increase in the accumulation of bacterial plaque (Zachrisson 1976). Previous studies have focused their attention on the effects of the placement of orthodontic appliances on the specific microbial composition of the subgingival plaque. An increase in motile organisms (Leggott et al. 1984) and black-pigmenting *Bacteroides* (Diamanti-Kipioti et al. 1987) has been described in subgingival plaque from patients wearing fixed orthodontic appliances. *Actinobacillus actinomycetemcomitans* (*Aa*) is an age-related microorganism (Slots et al. 1990) that is frequently associated with early-onset periodontitis (Slots & Schonfeld 1991). To date, however, only a few studies (Paolantonio et al. 1996, 1997, 1999) have monitored young periodontally healthy orthodontic patients for the presence of *Aa*, and noted a high frequency of detection as compared with orthodontic-appliance-free, matched-control individuals.

Gingival crevicular fluid (GCF) reflects the immune and inflammatory reactions deriving from host–parasite interactions (Lamster 1992; McCulloch 1994) and bio-mechanical stress (Griffiths et al. 1988; Grieve et al. 1994; Lowney et al. 1995; Uematsu et al. 1996). For this reason, many investigations have been able to demonstrate that GCF constituents, including microbial products, can be useful for the determination of the periodontal state during periodontal inflammation (McCulloch 1994) or orthodontic tooth movement (Perinetti et al. 2002). However, most of these studies have been aimed at determining whether any GCF constituent or crevicular microbiota can have potential uses in the diagnosis of periodontal diseases (Lamster 1992; McCulloch 1994). Thus, relatively few studies have investigated the changes in the levels of GCF constituents or host–parasite colonization during orthodontic tooth movement (Uematsu et al. 1996; Paolantonio et al. 1999; Perinetti et al. 2002).

It has been demonstrated that an inflammatory host response can lead to tissue remodelling and allow tooth movement (Grieve et al. 1994; Lowney et al. 1995; Uematsu et al. 1996). Through animal models, responses of bone tissue to tension or compression forces have also been reported (Rygh 1972, 1976; King et al. 1991). Previous in vitro investigations have studied the responses of various periodontal cells to mechanical stimuli (Ozawa et al. 1990; Yamaguchi et al. 1996). In terms of the GCF metabolites, Uematsu et al. (1996) have shown that during orthodontic treatment the levels of different inflammatory mediators, such as interleukine-1β (IL-1β), interleukine-6, tumour necrosis factor-α (TNF-α), epidermal growth factor and β_2 microglobulin, can undergo significant elevations. Grieve et al. (1994) have obtained similar results for prostaglandin E and IL1-β, while Lowney et al. (1995) have described an increase in TNF-α in GCF from teeth undergoing orthodontic force. Griffiths et al. (1988) have reported an elevation of osteocalcin and pyridinium crosslinks of bone collagen in GCF from orthodontically treated teeth.

Alkaline phosphatase (ALP) is a host enzyme that allows bone deposition by hydrolysing inorganic pyrophosphate, which is a potent inhibitor of the mineralization process (Robinson 1923). Periodontal cells, such as fibroblasts of the periodontal ligament (PDL) and osteoblasts of the alveolar bone, have ALP activity (King et al. 1991; Keeling et al. 1992; Yamaguchi et al. 1996). Aspartate aminotransferase (AST) is an enzyme normally confined to the cytoplasm of cells and its increased extracellular levels are caused by cell membrane lysis after necrosis (Williams & Marks 1983). However, basal levels of AST in the extracellular environment can be detected as a consequence of physiological tissue turn-over (Williams & Marks 1983). Both ALP and AST activities have been shown to be present in human GCF (Ishikawa et al. 1970; Chambers et al. 1984). Indeed, several studies have evaluated the GCF ALP and AST activity levels during periodontal disease (Ishikawa et al. 1970; Persson et al. 1990; Nakashima et al. 1994). ALP has been extensively correlated with the rate of bone formation (Christenson 1997), while both these enzymes appear to undergo significant elevation in GCF if surrounded by inflamed periodontal tissues (Ishikawa et al. 1970; Persson et al. 1990). To date, only a longitudinal study (Perinetti et al. 2002) has been performed in order to evaluate GCF ALP activity changes during orthodontic tooth movement in humans, while similar evidence regarding GCF AST is still lacking. This controlled longitudinal study was aimed at evaluating *Aa* colonization changes in the subgingival plaque with the concomitant evaluation of GCF ALP and AST activity changes at the same sites in young healthy subjects undergoing orthodontic treatment. Moreover, the different stresses exerted on the periodontium (tension or compression) by the tooth movement have also been taken into account.

Materials and Methods

Study population

Twenty-one orthodontic patients (11 females and 10 males; ages: 11.2–22.5; mean 17.1 ± 3.3 years) who presented themselves to the Unit of Orthodontics, Department of Oral Sciences, ''G.

D'Annunzio'' University, and were diagnosed for the extraction of their first premolars because of dental crowding participated in this study. The following criteria were observed in the enrolment of the patients: (1) the need for fixed appliance therapy involving distal retraction of one maxillary canine; (2) a healthy systemic condition; (3) no use of anti-inflammatory drugs in the month preceding the beginning of the study; (4) probing depth (PD) values, measured as the distance from the bottom of the sulcus to the most apical portion of the gingival margin, not exceeding 4 mm in the whole dentition; (5) no loss of periodontal attachment, measured as the distance from the bottom of the sulcus to the cemento-enamel junction, exceeding 2 mm in any interproximal site; (6) no radiographic evidence of periodontal bone loss after a full-mouth radiographic periapical examination; and (7) a full-mouth plaque score (FMPS) and a full-mouth bleeding score (FMBS) $\leqslant 20\%$. FMPS and FMBS were recorded as the percentage of tooth surfaces with the presence of supragingival plaque or bleeding within 15 s after probing with a $20 \times g$ controlled-force probe (Vivacare TPS Probe, Vivadent, Schaan, Lichtenstein). During the 2 months preceding the baseline examination, all subjects received repeated oral hygiene instructions (OHIs) about the correct use of a toothbrush, dental floss and an interdental brush, and 2 weeks before the baseline examination all patients underwent one session of meticulous supra- and subgingival ultrasonic scaling. Moreover, the study subjects were not allowed to take either anti-inflammatory drugs or mouthwashes containing chlorhexidine for the entire duration of the study, due to their potential of affecting the results (Uematsu et al. 1996). Informed consent was obtained from the patients and the parents of patients under 18 years of age prior to the commencement of the study, and the protocol was reviewed and approved by the Ethical Committee of the G. D'Annunzio University Medical Faculty.

Orthodontic appliance

In each participant, 2 months after the maxillary premolar extractions, one maxillary canine (DC) was distalized and considered as the test tooth, while the contralateral canine (CC) and antagonist canine (AC) were used as controls. On the maxillary arch of each subject, orthodontic brackets (MBT, 3M-Unitek, Monrovia, CA, USA) were placed on the buccal surfaces of the teeth, including the incisors, canines and premolars; bands were also placed on the first molars. A monolateral passive arch wire was then fixed to this appliance. The maxillary canine wearing the monolateral arch was the DC. At the same time, a nickel titanium open-coil spring (American Orthodontics, Sheboygan, WI, USA) exerting a constant force of $150 \times g$ was included in the appliance in order to move the DC distally. This coil spring was compressed between the lateral incisors and the DC. The entire orthodontic appliance was placed in a single clinical session. No orthodontic appliance was placed on the mandibular arch. Further OHIs were given to the subjects in order to inform them regarding how to perform effective tooth cleaning in the presence of the orthodontic appliance.

Clinical monitoring and sampling procedures

The clinical examination was performed on the corresponding sampling sites situated mesially and distally on each DC, CC and AC. This consisted of recording the presence of supragingival plaque (PL+), assessed by visual criteria, and assessing both gingival bleeding within 15 s after probing with a $20 \times g$ controlled force probe (BOP+), and the PD. The same operator (D. D'A.) always collected the clinical data. Contamination of the GCF and subgingival plaque samples was minimized by recording the PL+ before carefully cleaning the tooth with a sterile curette, collecting GCF and subgingival plaque from the isolated area, and then recording the PD and BOP+. The GCF and subgingival plaque samplings were performed after the sites had been isolated with cotton rolls and dried by a gentle air stream. Then three #30 standardized sterile paper strips (Inline, Torino, Italy) were inserted 1 mm into the gingival crevice and left in situ for 10 s. One of these was used for the bacteriological examination, while the others were used for the ALP and AST activity assays. The clinical parameter recordings and GCF and subgingival plaque samplings were performed twice: at baseline (prior to orthodontic appliance placement) and on day 28. Verification of dental movement was determined as previously described (Perinetti et al. 2002).

Bacteriological methods

Immediately after collection, each microbial sample was inserted into a vial containing 0.5 ml of reduced transport fluid (RTF) without EDTA. The vials were flooded with nitrogen and transported to the bacteriological laboratory within 40 min, where they were placed into an anaerobic chamber containing an atmosphere of 10% CO_2, 10% H_2 and 80% N_2 (Don Witley Scientific Ltd; International PBI spa, Milan, Italy). The plaque suspension was dispersed by mild sonication for 10 s under anaerobic conditions and serially diluted in RTF. Aliquots of 0.1 ml of the appropriate dilution were spread onto trypticase soy–serum–bacitracin–vancomycin (TSBV) agar plates, a selective medium for *Aa* (Slots 1982), and onto non-selective enriched trypticase soy agar (ETSA) plates for total anaerobic colony counts.

The inoculated selective and non-selective media were then cultured at 37°C in a microaerophilic environment (5% CO_2, 95% N_2) and in an anaerobic chamber, respectively. After 7 days, the TSBV agar plates were examined for the presence of *Aa*. Small, translucent, slightly convex, circular colonies, often with a star-like inner structure and adherent to the agar surface, were subcultured for identification. A definitive identification was made on the basis of Gram stain, nitrate reduction, production of catalase, urease and indole, growth on McConkey agar and fermentation reactions to carbohydrates (fructose, glucose, lactose, maltose, mannitol, sucrose and xylose) supplemented by the profiles of preformed enzymes (API-ZIM System, BioMèrieux Italia SpA, Rome, Italy). Plates containing between 30 and 300 colony-forming units were selected for enumeration.

Enzymatic activity determinations

Both the ALP and AST activities were assayed spectrophotometrically (Karmen 1955; Bowers & McComb 1966) (Model 8453, Hewlett Packard, Waldgrohn, Germany), incubating the cone samples at 30°C (± 0.05°C) in 1 ml of substrate for 5 min in a 1-cm-path-length quartz cuvette. For the ALP assay, the substrate consisted of *p*-nitrophenyl phosphate (10 mM), carbonate buffer (pH 10.2 ± 0.1, at 30°C), mannitol (200 mM) and $MgCl_2$ (3 mM). ALP hydrolyses *p*-nitrophenyl phosphate to *p*-nitrophenol

and inorganic phosphate. The rate of increase in absorbance at 405 nm was monitored as the *p*-nitrophenol formed. Using 18.45 as the *p*-nitrophenol millimolar absorptivity, the absorbance was converted into enzyme activity units (1 U = 1 μmole of *p*-nitrophenol released per min at 30°C). For the AST assay, the substrate contained 150 mM L-aspartate, 100 mM 2-oxoglutarate, 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), 400 mU/ml malate dehydrogenase and 100 mM phosphate buffer (pH 7.4 $\pm$ 0.1, at 30°C). In the presence of AST, L-aspartate and 2-oxoglutarate exchange an amino group to yield oxalacetate and L-glutamate. The rate of this reaction was monitored by the use of an indicator reaction in which the oxalacetate formed is reduced to L-malate by an excess of malate dehydrogenase, with the simultaneous oxidation of NADH. The change in absorbance at 340 nm was monitored as the NADH was consumed. A value of 6.22 was considered as the NADH millimolar absorptivity in calculating enzyme activity units (1 U = 1 μmole of NAD^+ released per min at 30°C). The final results are expressed as total ALP/AST activity (mU/sample).

Data processing

The Statistical Package for Social Sciences program (SPSS® Inc., Chicago, IL, USA) was used to perform the data analysis. PL+ and BOP+ were considered as the number of experimental sites positive for the presence of supragingival plaque and bleeding on probing, and therefore these scores were treated as dichotomous data. A Cochran test followed by a Bonferroni-corrected McNemar test, when appropriate, were employed to assess the significance of differences in the PL+ and BOP+ among the experimental groups; in the same way, the significance of changes over time within the groups was assessed by the McNemar test. Each data set (PD, *Aa* colonization levels, GCF ALP and AST activities) was tested for the normality of the data by means of the Shapiro–Wilk test and by *Q–Q* normality plots, and equality of variance by means of the Levene test and *Q–Q* normality plots of the residuals. Most of the data sets failed to meet the required assumptions for using parametric methods; hence, alternative, non-parametric methods were performed in the hypothesis testing. For all these parameters, a Friedman test followed by a Bonferroni-corrected Wilcoxon rank-sum test, where appropriate, were used to assess the significance of differences among the experimental groups, at the baseline and at the 28-day examinations, by site. Moreover, the Wilcoxon paired sign-rank test was also performed in order to analyse the significance of differences within each experimental tooth by site over time, and between sites within each experimental tooth by time point. A p value less than 0.05 was used for rejection of the null hypothesis.

Results

Clinical parameters

The DCs underwent a mean distal movement of 1.8 $\pm$ 0.7 mm. No dental displacement was seen in the CC and AC groups. As reported in Table 1, clinical parameter scores at baseline were similar, without statistically significant differences, irrespective of the experimental groups or sites. Longitudinal examinations showed that on day 28 the number of sites positive for supragingival plaque (sites PL+) and bleeding on probing (sites BOP+), and the mean PD were significantly increased, as compared with baseline, in both the DC and CC mesial and distal sites; the same was not observed in the ACs, where all clinical parameters remained unchanged throughout the study. On day 28, significant differences were observed for the clinical parameters among the three groups, in both the mesial and distal sites, for all clinical parameters. Of note, the results of pair-wise comparisons show no significant differences in any of the clinical parameters between the DCs and the CCs. However, in distal sites, significantly greater scores for PL+ and BOP+ between the DCs and the ACs, and for BOP+ between the CCs and the ACs, were detected. The data for the mean PD in both mesial and distal sites demonstrated significantly greater values between the DCs and the ACs, and between the CCs and the ACs. Moreover, at the same clinical session, no significant differences between mesial and distal sites within each experimental group were observed.

Microbiological and enzymatic parameters

The microbiological and enzymatic data are summarized in Table 2.

At baseline, *Aa* was not isolated from any of the experimental sites of the 21 subjects. On day 28, *Aa* was isolated from 11 DCs, both at mesial and distal sites, from 13 mesial CC sites, from 12 distal CC sites and from one distal AC site. The mean proportions of *Aa* significantly increased from baseline to day 28 in each experimental site of the DCs and the CCs, while they did not change significantly throughout the entire study in the ACs. In particular, on day 28 these proportions were higher in all the DC and CC sites, as compared with those of the ACs. No significant differences were found for the *Aa* colonization between the DCs and the CCs at mesial and distal sites, nor between mesial and distal sites within the same experimental tooth.

At the baseline, in both mesial and distal sites the GCF ALP activity was similar among the three groups, with no significant differences. The GCF ALP activity significantly increased over time in both the mesial and the distal DC sites and the mesial CC sites. In the distal CC sites, an ALP activity increase, although not significant, yielded a p value of 0.051. Finally, in the ACs, this enzymatic activity was stable throughout the study, without any statistically significant changes. On day 28, enzymatic activity was significantly greater in the DCs, as compared with the CCs and the ACs in mesial sites, and as compared with the ACs in distal sites; moreover, this activity was greater in both the mesial and the distal CC sites, as compared with the corresponding AC sites. Furthermore, at this examination, a significantly greater GCF ALP activity within the DCs was found in the mesial sites (tension) in comparison with the distal sites (compression).

Similar to the data for GCF ALP, the AST activity at baseline was the same among the experimental groups, and significant increases were seen over time in all the DC and the CC sites, while no significant changes were detected in the AC group on day 28 as compared with the baseline. On day 28, this enzymatic activity was significantly greater in the DCs, as compared with the CCs and the ACs in distal sites, and as compared with the ACs in mesial sites; moreover, the GCF AST activity

Table 1. Clinical parameter changes of the different groups between the baseline and the 28-day examination ($n = 21$)

Time	DCs		CCs		ACs		Among groups differences		
	mesial	distal	mesial	distal	mesial	distal	mesial	distal	total
PL+									
baseline	4	5	4	5	3	3	–	–	NS
28 days	12	11a	10	12	4	3	$p<0.05$	$p<0.05$	$p<0.01$
diff.	$p<0.05$	$p<0.05$	$p<0.05$	$p<0.05$	NS	NS			
BOP+									
baseline	4	3	2	4	3	3	–	–	NS
28 days	11	11a	9	12a	3	2	$p<0.05$	$p<0.01$	$p<0.01$
diff.	$p<0.05$	$p<0.05$	$p<0.05$	$p<0.05$	NS	NS			
PD									
baseline	1.5±0.6	1.3±0.5	1.5±0.7	1.7±0.6	1.6±0.6	1.5±0.7	–	–	NS
28 days	2.4±0.8a	2.3±0.7a	2.4±0.9a	2.1±0.7a	1.7±0.7	1.5±0.5	$p=0.01$	$p<0.01$	$p<0.01$
diff.	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.05$	NS	NS			

Results of pair-wise comparisons among the groups at the 28-day examination. PL+, number of gingival sites positive for the presence of bacterial plaque; BOP+, number of gingival sites positive for the presence of bleeding on probing; PD, mean values±SDs of probing depth. diff, statistical significance of differences over time; a, significantly different from the corresponding site of the ACs; NS, no statistically significant difference.

Table 2. Aa subgingival colonization, and GCF ALP and AST activities of the different groups between the baseline and the 28-day examination ($n = 21$)

Time	DCs		CCs		ACs		Among groups differences		
	mesial	distal	mesial	distal	mesial	distal	mesial	distal	total
Aa									
baseline	ND	ND	ND	ND	ND	ND	–	–	–
28 days	0.59±0.23a	0.56±0.21a	0.51±0.21a	0.46±0.18a	ND	0.01±0.00	$p<0.01$	$p<0.01$	$p<0.01$
diff.	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	NS	NS			
GCF ALP									
baseline	60±36	52±18	59±33	61±23	46±26	43±19	–	–	NS
28 days*	159±83a,c	102±43a	80±31a	81±26a	47±24	44±24	$p<0.01$	$p<0.01$	$p<0.01$
diff.	$p<0.01$	$p<0.01$	$p<0.05$	NS	NS	NS			
GCF AST									
baseline	168±64	209±113	177±98	162±95	196±111	198±112	–	–	NS
28 days	328±145a	423±186a,c	225±92a	267±116a	162±33	163±116	$p<0.01$	$p<0.01$	$p<0.01$
diff.	$p<0.01$	$p<0.01$	$p<0.05$	$p<0.05$	NS	NS			

Aa: *Actinobacillus actinomycetemcomitans*; GCF: gingival crevicular fluid; ALP: alkaline phosphatase; AST: aspartate aminotransferase.
Results of pair-wise comparisons among the groups at the 28-day examination. *Aa* levels are presented as mean±SE of proportions of colonization. ND, levels not detectable. GCF ALP and AST activities are presented as means±SDs of total activity (mU/sample). diff, statistical significance of differences over time; a and c, significantly different from the corresponding site of the ACs and the CCs, respectively.
*Within the DCs, statistically significant difference in the GCF ALP activity between mesial and distal sites ($p<0.01$). NS, no statistically significant difference.

was greater in both the mesial and the distal CC sites in comparison with the corresponding AC sites.

Discussion

In the present study, subgingival colonization of *Aa* and GCF ALP and AST activities were longitudinally monitored in human subjects in order to evaluate both the early microbiological and host responses to orthodontic treatment. Moreover, the effects of two different types of mechanical stress on the periodontium, tension and compression were also evaluated. The results demonstrate that at the 28-day observation, there were detectable increases in all evaluated parameters in the DCs and the CCs, as compared with the ACs. No differences were detected between the DC and the CC groups for the colonization of *Aa*. On the contrary, the GCF ALP and AST activities were greater in the DCs, as compared with the CCs, in the mesial (tension) and distal (compression) sites, respectively. Furthermore, within the DCs at the 28-day examination, the GCF ALP activity was greater in the tension sites as compared with those of compression.

A clinically detectable dental displacement was seen in the DC group only. As several studies have reported (Zachrisson 1976; Trossello & Gianelly 1979), during the fixed appliance orthodontic stage an increase in the plaque accumulation and gingival inflammation is frequently seen. Other studies have demonstrated that an adequate oral hygiene programme, performed before and during the orthodontic treatment, can minimize the increase in plaque accumulation (Lundstrom & Hamp 1980; Lundstrom et al. 1980). In this study, in spite of accurate OHIs being given to all subjects, a worsening of the periodontal conditions of the teeth included in the orthodontic fixed appliance was present after 28 days of treatment (Table 1).

This can be attributed to the increased difficulty of effective cleaning around the appliance. Moreover, a significant increase in PD of the DCs and the CCs was seen. This change could be more properly related to a moderate gingival enlargement, without periodontal attachment changes, rather than attachment loss; nevertheless, the *Aa* colonization increased. This hypothesis is supported by a previous study (Paolantonio et al. 1997) carried out in young orthodontic patients within 3 years of follow-up, which found that the presence of *Aa* in subgingival plaque did not represent a significant risk factor for the occurrence of periodontal attachment loss; indeed, the virulence of the bacterium depends on several bacterial serotypes (Zambon et al. 1983; Di Rienzo & Slots 1990) and on the host's individual susceptibility (Slots & Schonfeld 1991; Socransky & Haffajee 1992). Finally, in the presence of fixed orthodontic appliances, such gingival enlargement has been reported previously (Zachrisson & Zachrisson 1972; Kloehn & Pfeifer 1974; Alexander 1991).

At baseline, none of the subjects were positive for the presence of the bacterium under consideration; this was probably because of the session of careful professional oral hygiene 2 weeks before the beginning of the study. Conversely, at the end of the study *Aa* was isolated in 13 out of 21 subjects (62%). This level of *Aa* detection is similar to that reported in previous studies (Paolantonio et al. 1996, 1999). The increase in the colonization of *Aa* in the subgingival plaque of DCs and CCs is not surprising since previous cross-sectional (Paolantonio et al. 1996) and longitudinal (Paolantonio et al. 1997) studies have documented such modifications in patients wearing fixed orthodontic appliances (Paolantonio et al. 1999). Moreover, no statistically significant differences in colonization of *Aa* were found between mesial and distal sites of both the DCs and the CCs. The increase in *Aa* detection can be attributed to the worsening of the clinical parameters (Paolantonio et al. 1999) (Table 1); indeed, *Aa* is reported to be more often detectable in the subgingival plaque when a high amount of supragingival plaque is also present (Wolff et al. 1985). Conversely, other observations have reported large increases in anaerobic subgingival flora, although no changes in the supragingival plaque scores occurred in these orthodontic patients (Diamanti-Kipioti et al. 1987). The results obtained in the present study are in agreement with those reported by Paolantonio et al. (1999), who observed increases in *Aa* in the subgingival plaque of orthodontically treated patients longitudinally monitored; they also found a persistence of elevated colonization of *Aa* in the subgingival plaque of teeth after a 1-month rest period following 3 months of fixed orthodontic appliance treatment. Finally, *Aa* subgingival colonization between mesial and distal sites within both the DCs and the CCs was the same. This suggests that the *Aa* colonization probably depends on the plaque accumulation after orthodontic appliance placing upon the teeth, rather than the orthodontic force.

GCF ALP activity was increased in the DCs and the CCs as compared with the ACs. Several reports have described significant correlations between GCF ALP activity and gingival inflammation (Nakashima et al. 1994). These data support the increased enzymatic activity found in the CCs since this experimental tooth was free from any mechanical stress, but showed a significant increase in all the clinical parameter scores recorded at the 28-day examination as compared with those of the baseline (Table 1). The same worsening of the clinical conditions reported in the DCs could be involved, in part, in the GCF ALP activity increase in this experimental tooth. The enzymatic activity in the DCs was greater as compared with the CCs. This difference was probably due to the orthodontic tooth movement seen for the DCs. Indeed, ALP is considered to be a marker for bone deposition (Rodan 1991), and several studies have reported changes in the ALP activity in osteoblasts of the alveolar bone during experimental tooth movement in rat models (Takimoto et al. 1968; Keeling et al. 1992). A previous report (Insoft et al. 1996) also described an increase in GCF ALP activity in humans during orthodontic tooth movement; however, this study was based on a cross-sectional design on patients undergoing orthodontic treatment from 6 to 24 months. We have found a remarkable increase in GCF ALP activity after 4 weeks of orthodontic force upon the teeth. This evidence is supported by other investigations that have evaluated the alveolar bone metabolism changes undergoing mechanical stress in a rat model. In particular, these authors (King et al. 1991; Keeling et al. 1992) hypothesized an early wave of resorption, which requires 3–5 days, which is followed by its reversal (5–7 days), and by a late wave of bone formation that continues for 7–14 days. This process appears to occur on both pressure and tension sides of the alveolar wall (King et al. 1991). Moreover, a similar process has been described for human bone (Rodan 1991; Christenson 1997). In particular, in humans the bone formation appears to begin after 10 days (Christenson 1997) or 3 weeks (Frost 1991, 1992). Considering that in the phase of bone formation the ALP activity has been reported to be increased as compared with other phases (Rodan 1991), the GCF ALP activity found in the DCs after 4 weeks of treatment can be attributed to the bone formation in the alveolar bone (King et al. 1991). Finally, the greater enzymatic activity found in the tension site, as compared with that of compression, can be explained by the prevalence of bone deposition over resorption (Rygh 1976). An increase in GCF ALP activity during orthodontic tooth movement appears to occur from 1 to 4 weeks of treatment, as we have described previously (Perinetti et al., 2002); however, in that study a control tooth characterized by the presence of an orthodontic appliance without mechanical stress (CCs) was not considered. It is of interest that while the worsening of clinical parameters could be contributed to GCF ALP activity increases (Nakashima et al. 1994), as these were similar between the tension and compression sites within the DCs (Table 1), it is unlikely that these could lead to the greater enzymatic activity recorded in the tension sites of the DCs.

AST is an intracytoplasmic enzyme that is released into the external environment after cell membrane rupture (Williams & Marks 1983). For this reason, this enzyme is considered to be a good indicator of cell necrosis, and its serum activity levels are used to provide information regarding recent myocardial infarction or other conditions related to massive tissue destruction (Schmidt & Schmidt 1985). In recent years, many investigations have demonstrated that the AST activity in the GCF can be used as a diagnostic aid during periodontitis (McCulloch 1994; Magnusson et al. 1996) or peri-implantitis (Paolantonio et al. 2000). Indeed, it has been clearly shown that this enzyme

activity in the GCF is associated with tissue destruction that occurs during experimental gingivitis (Chambers et al. 1984) or chronic adult periodontitis (Persson et al. 1990). It has even been proposed that GCF AST activity can be considered to be a good predictor of tissue destruction during chronic adult periodontitis (Persson et al. 1990). A chairside kit for GCF AST activity determination is also available now (Magnusson et al. 1996). The present study has evaluated the possible role of GCF AST during the early events that lead to orthodontic tooth movement in humans. The greater enzymatic activity found in the DCs and the CCs can be explained by the worsening of the gingival conditions recorded for these experimental teeth (McCulloch 1994). Moreover, GCF AST activity may also be related to the increases in *Aa* colonization levels found in these experimental teeth, as compared with the ACs; indeed, a positive correlation between the *Aa* levels and GCF AST activity has been described previously (Kuru et al. 1999). However, the highest values of enzymatic activity in the DCs were presumably also due to the tissue modifications induced by the tooth movement recorded in this group. Indeed, a hyaline zone characterized by cell necrosis has been described in the PDL surrounding orthodontically moved teeth (Rygh 1972, 1976). This zone has been described as an aseptic focal necrosis that occurs in the compressed areas (Davidovitch et al. 1988). Of note, the persistence of this hyaline zone depends on the magnitude of the force (Lilja et al. 1983). Although the studies mentioned above were based on animal models, presumably such a hyaline zone also occurs in the human PDL in vivo, from where an increased release of AST into the extracellular environment occurs. Since such necrosis has been described to be present only in compression areas, this may only explain the significant GCF AST activity increase in the compression sites of the DCs, as compared with that recorded in the CCs. However, in the present study, no significant differences were found between tension or compression sites, neither for the clinical parameters nor for GCF AST activity. However, as reported above during orthodontic treatment, the bone metabolism changes that allow tooth movement are characterized by a combination of tissue deposition and resorption in both tension and compression sites (King et al. 1991). According to these findings, significant tissue destruction may have been present in the tension sites of the DCs at the 28-day monitoring, causing an increase in GCF AST activity in the same site.

Conversely, in the ACs, where no tooth movement occurred and where there was no orthodontic appliance that could interfere with gingival health, all the evaluated parameters remained at baseline values during the entire experimental term.

In brief, within the limitations of our study, the results confirm that significant subgingival microbiological changes can occur as a consequence of plaque accumulation after orthodontic appliance placement. Although GCF AST may be considered to be an indicator of tissue modifications during orthodontic tooth movement in humans, it appears not to be as sensitive as GCF ALP in distinguishing between tension and compression sites 28 days after the beginning of the treatment. However, other factors, such as clinical condition changes, may affect the enzymatic activities; hence, the GCF ALP and AST should be considered as reliable biomarkers of tissue responses to orthodontic tooth movement in humans only when oral hygiene is kept under control.

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