Scientific Article

The composition of subgingival microflora in two groups of children with and without primary dentition alveolar bone loss

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Abstract

The is study examined the relationships between the microbial composition of the subgingival plaque, contact loss caused by caries and alveolar bone loss (ABL) in primary molars. The study included 10 children with contact loss in at least two sites, one with ABL and one without ABL, and 10 children without ABL with sites with or without contact loss. The microbial composition of subgingival plaque was examined by dark-field microscopy and by cultures of total anaerobic bacteria, Actinobacillus actinomycetemcomitans (Aa) *and* Porphyromonas gingivalis (Pg). *Dark-field microscopy confirmed that spirochetes and motile rods may be part of the indigenous flora of the oral cavity. More spirochetes and motile rods were observed in sites with ABL than in control sites in the same subject and control subjects without ABL. Lower numbers of cocci were seen in sites with ABL than in sites in children without ABL, but a significant difference was not observed between sites with ABL and healthy sites within the same subjects. No significant differences in the dark-field values were evident in sites without ABL, with or without contact loss. Aa and Pg were found in children and sites with or without ABL. In sites with Aa, larger proportions of spirochetes, lower values of cocci, and more colonies of Pg were evident. No significant differences in anaerobic bacteria were evident between sites with or without contact loss or with or without ABL. ABL in the primary dentition was found to be related to the microbial composition of the subgingival plaque, but not related to contact loss per se. (Pediatr Dent 18:42–47, 1996)*

The possible relationship between alveolar bone loss (ABL) in childhood and adulthood in the same individual has emphasized the need to study the etiology of ABL in children.[1–3] In addition, marginal ABL in the primary dentition adjacent to extensive proximal caries, which facilitates food impaction and plaque accumulation, has been described in several studies.[4–8] The facts that marginal ABL does not take place in every child with extensive proximal caries, and that in children affected with ABL the defect tends to appear in more than one site, suggest that some children may have an increased susceptibility to marginal ABL.[4–6]

Identifying susceptible children, as well as the factors that make them more susceptible to ABL, may lead to preventive measures directed at reducing the deleterious effect of modifiable risk factors, such as the bacterial composition of the subgingival dental plaque and host-related factors.[3, 9–12] The purpose of this study was to assess, in the primary molar area, the relationships between the microbial composition of the subgingival plaque, contact loss caused by caries, and alveolar bone loss.

Methods and materials

Following approval by the institution's Helsinki Committee, this study was conducted at the Pediatric Dentistry Clinic of the Hebrew University — Hadassah School of Dental Medicine. Children who had no systemic diseases, had not received antibiotic treatment for at least 2 months, and had good quality bitewing radiographs were considered as potential subjects for the study. Good quality radiographs were determined as having 1) minimal or no distortion, 2) minimal or no overlapping between the proximal surfaces of the primary molars, and 3) a clear image of the alveolar bone area.

The first 20 children who fit the following groups (10 in each group) were included in the study:

Group A: Children with contact loss due to proximal caries between the primary molars, with at least one site with ABL and one site without ABL.

Group B: Children with no ABL, and with sites with or without contact loss due to proximal caries.

The alveolar bone was considered normal when the distance from the cementoenamel junction to the alveolar bone crest (CEJ-ABC distance) was ≤ 2 mm and/or the lamina dura was complete. Marginal ABL was defined when the CEJ-ABC distance was > 2 mm and there was clear evidence of the lamina dura being com-

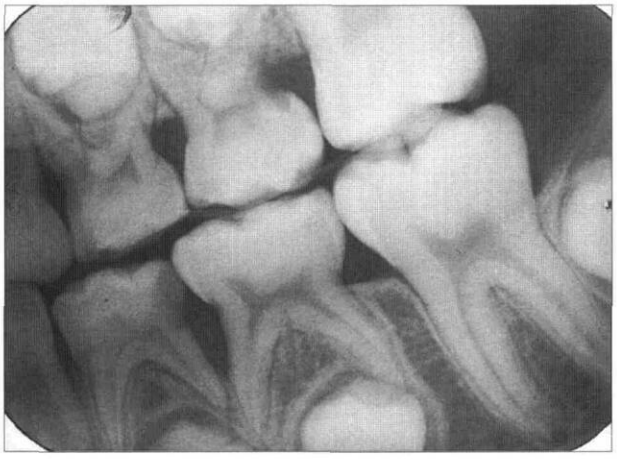

Fig 1. Bite-wing radiograph in which alveolar bone loss is evident between the lower primary molars with contact loss due to caries.

pletely absent (Fig 1). The CEJ-ABC measurements were performed on the bite-wing radiographs using a light box and an optical device (Loupe x10) composed of a magnifying glass and a micro-ruler. The device allowed for measurements in 0.1-mm increments.

Subject collection was done within 8 weeks. The sites with contact loss were not yet restored at the time of the subgingival plaque sampling. Two samples of the subgingival plaque were obtained from each child in group A, one from a site with ABL (subgroup A-1) and the other from a site without ABL (subgroup A-2). In group B, an attempt was made to obtain an equal number of samples with or without contact loss from each child. However, due to the distribution and severity of caries, seven samples were obtained from sites with contact loss (subgroup B-1) and 13 from sites without contact loss (subgroup B-2).

For the microbial examination, supragingival plaque was removed and the teeth carefully dried. Three sterile paper points then were inserted for 10 sec to the base of the sulcus or pocket, removed, and immediately inserted into a vial containing 0.3 ml of reduced transfer fluid (RTF).[13] The samples were vortexed for 60 sec and then serially diluted in RTF (1:100, 1:200).

For dark-field microscopy, 30 μl of the sample was taken and diluted 1:1 with sterile saline with 2% gelatin. The following microorganisms were counted and their percentages from a total of 100 were calculated: filaments, cocci, rods, spirochetes, and motile rods. The total percentages for nonpathogenic-related (filaments, rods, and cocci) and pathogenic-related microorganisms (spirochetes and motile rods) also were calculated.[14-17]

Samples from each dilution were plated by means of an automatic spiral platter[18] on:

1. Enriched trypticase soy agar (ETSA) for the total anaerobic count.[19]
2. Trypticase soy bacitracin vancomycin (TSBV) for *A. actinomycetemcomitans* (Aa).[20] Only catalase-positive bacteria with the specific typical star-shaped colony were counted as Aa.
3. Bacteroides gingivalis agar (BGA) for *Porphyromonas gingivalis* (Pg).[21]

Plates from each sample then were incubated to assess the anaerobic organisms in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) for 7 days in an atmosphere of N_2, H_2 and CO_2 at 37°C.

Following incubation, the total number of colony forming units (CFU) was identified and counted by means of colony morphology. The mean value for two plates per site for each type of bacteria and their proportions from the total anaerobic counts were calculated.

Statistical analysis

The student's *t*-test was utilized to examine the significance of the difference in: age between groups A and B; CFU and dark-field values for sites with or without Aa and with or without Pg with or without marginal ABL. Analysis of variance (ANOVA) was utilized to examine the significance of the differences in CEJ-ABC distances, dark-field microscopy, and CFU values between groups A and B and among the four subgroups. The Fischer PLSD was used to evaluate the statistical significance of the differences between pairs of subgroups. A standard computer program for statistical analysis was utilized for the statistical examination (StatView™ SE+Graphics, Abacus Concepts, Inc, Berkeley, CA). Differences at the 5% level of probability were considered statistically significant.

Results

The children's ages ranged from 6 to 10 years. No statistically significant difference in age was found between group A and group B (N = 10, mean = 8.1 years, SD = 1.3 and N = 10, mean 7.5 years, SD = 1.0, respectively).

The overall differences between the CEJ-ABC distances of the four subgroups were statistically significant (ANOVA, $P = 0.0001$). Significant larger CEJ-ABC distances were evident in the sites with ABL (subgroup A-1) than in all the other subgroups (Fisher PLSD $P < 0.05$). Among the children without ABL (group B), larger but not statistically significant CEJ-ABC distances were found in sites with contact loss (subgroup

TABLE 1. MEAN DISTANCES IN MM FROM THE CEMENTOENAMEL JUNCTION TO THE ALVEOLAR BONE CREST IN CHILDREN WITH (GROUP A) AND WITHOUT (GROUP B) ALVEOLAR BONE LOSS (ABL)

Group	*Subgroup/Sites*	*N**	*Mean*	*SD*
A	1- with ABL	10	2.7	0.3
A	2- without ABL	10	1.5	0.5
B	1- with contact loss	7	1.5	1.1
B	2- without contact loss	13	1.0	0.5

* Number of sites.

TABLE 2. MEAN PROPORTIONS OF BACTERIAL MORPHOLOGIES OBTAINED WITH DARKFIELD MICROSCOPY

											Sum of			
	Filaments		*Cocci*		*Rods*		*Spirochaetes*		*Motile Rods*		*Nonpathogenic*		*Pathogenic*	
Group	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
A all	18.7	11.3	33.0	17.9	23.2	12.1	10.5	9.0	14.7	9.1	74.9	15.9	25.2	15.2
B all	17.5	6.7	48.6	14.7	24.0	11.3	5.6	4.8	4.4	5.7	90.1	9.3	10.0	9.3
P•	NS		0.006		NS		0.04		0.0001		0.0009		0.0006	
Subgroup														
A1	14.4	8.3	30.0	15.0	20.7	11.8	14.2	10.5	20.2	8.3	65.1	14.6	34.4	13.9
A2	23.0	12.7	36.0	20.9	25.8	12.6	6.8	5.6	9.1	6.0	84.8	10.3	15.9	10.1
B1	15.8	6.7	49.7	11.7	24.7	9.4	4.3	3.1	5.4	5.8	90.3	8.7	9.7	8.6
B2	18.4	6.7	47.9	16.5	23.5	12.5	6.3	5.4	3.8	5.7	89.9	10.0	10.1	10.0
P•	NS		0.04		NS		0.02		0.0001		0.0001		0.0001	

• ANOVA.

B-1) than in those without contact loss (subgroup B-2; Table 1).

Dark-field microscopy

Eighteen samples from nine children in group A (two samples from one child were damaged during processing) and 20 samples from 10 children in group B were examined.

All the examined bacteria were evident in the subgingival plaque of all children of group A. Among the group B children, spirochetes were not found in one child and motile rods were not found in four children.

Comparison of the mean proportions of bacterial morphologies between children in groups A and B and the children in the four subgroups (Table 2) indicated that group A had significantly lower mean proportions of cocci and the sum of nonpathogenic microorganisms (filaments, cocci, and rods), and significantly higher proportions of spirochetes, motile rods, and the sum of pathogenic microorganisms (spirochetes and motile rods).

ANOVA of the proportions of the different bacterial morphologies composing the subgingival plaque in the four subgroups (Table 2) indicated that the overall differences between the four subgroups were significant for cocci, spirochetes motile rods, and for the sums of nonpathogenic and pathogenic microorganisms. Analyses of the significance of the differences between pairs of subgroups (Fisher PLSD) revealed the following significant differences:

1. Among children with bone loss (group A), a lower proportion of filaments in sites with bone loss (subgroup A-1) than in sites without bone loss (subgroup A-2)
2. A lower proportion of cocci in sites with ABL (subgroup A-1) than in both subgroups of the children without bone loss (subgroups B-1 and B-2)

TABLE 3. MEAN VALUES OF COLONY FORMING UNITS OF TOTAL ANAEROBIC BACTERIA FROM SUBGINGIVAL PLAQUE OF CHILDREN WITH (A) AND WITHOUT (B) BONE LOSS

Group	*Subgroup/Sites*	*N*•	*Mean*	*SD*
A	1 = w/ABL	10	2.06×10^6	3.12×10^7
A	2 = w/out ABL	10	3.49×10^6	5.34×10^5
B	1 = w/contact loss	7	13.41×10^6	10.79×10^6
B	2 = w/*out contact loss*	13	13.16×10^6	12.71×10^6

• Number of sites.

3. Higher proportions of spirochetes, motile rods, and sum of pathogenic microorganisms in subgroup A-1 than in subgroups A-2, B-1, and B-2
4. A lower proportion of the sum of nonpathogenic microorganisms in subgroup A-1 than in subgroups A-2, B-1, and B-2.

Bacterial cultures

Total anaerobic bacteria

CFU of anaerobic bacteria were obtained from the plaque of all sites. No statistically significant differences were found between the mean total CFU values between the sites from group A (N = 20, mean = 2.78×10^6, SD = 4.3×10^6) and group B (N = 20, mean = 1.32×10^6, SD = 1.17×10^6), or among the four subgroups (Table 3).

Actinobacillus actinomycetemcomitans

CFU of *Aa* were obtained from the subgingival plaque from five sites of four children in group A (N = 5, mean proportion = 2.7%, SD = 2.3%) and from three sites of two children in group B (N = 3, mean proportion = 2.6%, SD = 4.2%). Due to the small number of sites from which Aa was obtained, no statistical analysis was done to analyze the differences among the groups/subgroups.

Comparison of the proportions of CFU of Pg and

dark-field values between sites with and without Aa revealed in sites with Aa:

1. A higher proportion of CFU of *Pg* (mean = 6.6%, SD = 11.2%, and mean = 1.9%, SD = 0.3%, respectively; $P = 0.01$)
2. Smaller proportions of cocci (mean = 30.0%, SD = 14.9%, and mean 44.1%, SD = 17.6% respectively; $P = 0.02$)
3. A higher proportion of spirochetes (mean = 11.7%, SD = 8.8%, and mean = 6.9%, SD = 6.8%, respectively; $P = 0.05$).

TABLE 4. MEAN PROPORTIONS OF CFU OF *P. GINGIVALIS* IN CHILDREN WITH (A) AND WITHOUT (B) ALVEOLAR BONE LOSS

Group	*Subgroup/Sites*	*N**	*Mean*	*SD*
A	1 = w/ABL	10	1.8	2.9
A	2 = w/out ABL	10	0.9	0.8
B	1 = w/contact loss	7	9.0	11.0
B	2 = w/out contact loss	13	1.8	2.5

* Number of sites.

Porphyromonas gingivalis

CFU of *Pg* were identified in the subgingival plaque of all 10 children of group A (18 sites) and in the plaque of seven children in group B (13 sites). The proportions of CFU of *Pg* from the CFU of total anaerobic bacteria are presented in Table 4. ANOVA revealed that the overall differences between the four subgroups were significant, and that subgroup B-1 had a significantly higher proportion of *Pg* than the other three subgroups. It should be noted, however, that these differences were the result of extremely high proportions of *Pg* in two sites with contact loss (15.9% and 30.9%) in only one child.

Comparison of the proportions of Aa and dark-field values between sites with and without Pg did not reveal any statistically significant differences.

Discussion

The diagnosis of ABL in children and adolescents is done mostly by radiographic examination.[4–8, 22–24] One should take into consideration, however, that radiographic evidence of widening of the periodontal ligament space, alveolar bone density, or an increase in the CEJ-ABC distances in the primary dentition, do not necessarily identify periodontitis.[25–28] On the other hand, a recent study indicated that radiographic evidence of a complete lack of lamina dura is indicative of ABL between the primary molars.[29] Accordingly, marginal ABL was recorded in this study only in sites with clear evidence of complete loss of the lamina dura, in addition to having a CEJ-ABC distance of > 2 mm.

Since bacteria and/or their products are the main etiologic factors of periodontal diseases,[9, 12] we examined the possible connection among the microbial composition of subgingival plaque, contact loss, and marginal ABL. Since the microbial composition of the subgingival plaque may change with age,[30] the similarity in age between the children in groups A and B allowed for comparison between the groups.

Our finding that most of the children — regardless of the presence/absence of ABL — had spirochetes and motile rods in their subgingival plaque, supports previous findings that indicate that these microorganisms are part of the indigenous flora of the oral cavity.[14, 30] Moreover, the increased percentages of spirochetes and motile rods in sites with ABL and the accordant higher percentages of cocci and rods in sites without ABL, support previous findings that related the presence of spirochetes and motile rods to gingivitis and periodontitis, and describe nonmotile bacteria as "healthy flora".[14–17]

Comparison of the present dark-field values for the children without ABL (48.6% cocci, 17.5% filaments, and 24.0% rods) with those of a previous study on teenagers with a mostly healthy periodontum (89.4% cocci, 4.5% rods, and 0.6% filaments)[17] reveals that in both studies the nonpathogenic component of the subgingival plaque was > 90%. The differences in the proportions of cocci, rods, and filaments between the studies may be due to population characteristics.

Interesting was the finding that in group B, no significant differences in dark-field values were evident in sites with or without contact loss. This finding suggests that contact loss per se does not facilitate ABL development, and that the presence of specific periodontopathogens and or host-related factors also are required for ABL development.

Aa and Pg, have been related to destructive periodontal disease, may colonize sites without clinical loss of periodontal attachment, and may be transmitted among family members.[12, 31–34] Our finding that Aa was found in children without ABL, is consistent with previous studies, which indicate that Aa may be found in individuals with no destructive periodontal disease.[30, 32, 34–36] In a previous study,[17] the dark-field microscopic data of Aa-positive subjects were similar to those of the Aa-negative subjects. In this study however, where the data were examined by site and not by subject, higher percentages of spirochetes and lower percentages of cocci were found in sites with Aa than in those without it.

In a previous study,[6] in which the bacterial composition of subgingival plaque from children with ABL was examined, Aa was identified in most of the affected sites. In this study however, Aa was identified only in three children of group A and two children in group B. The lower prevalence of Aa found in this study probably is related to the more accurate bacterial identification techniques utilized.

Pg has been described to be the most pathogenic and virulent of the black-pigmented Bacteroides species, which is rarely isolated from healthy young children and adolescents, and that is a major component of the subgingival flora in children with early-onset periodon-

titis.[30, 32, 37–38] On the other hand, detection methods such as DNA-probes and ELISA indicate that antibodies to Pg occur at low levels in most normal children and that Pg may be present in a significant part of the population below culture detection levels.[39–41] Therefore, it is possible that subgingival bacterial colonization of Pg during childhood and its transmission between family members is more prevalent than actually believed. The high prevalence of Pg found in this study among children with or without ABL may be due to the use of different selective media, and/or to population characteristics. In general, despite identification of Aa and Pg based on the use of selective media and colony shape utilized in several studies,[6, 12, 19, 20, 21, 32, 33, 35] one should consider superiority of DNA probes, which provide more accurate bacterial identification.[32, 39–41]

While our finding does not directly relate Aa or Pg to marginal ABL, there is still the possibility that Aa or Pg may flourish in neglected, marginal ABL lesions. Furthermore, one should take into consideration that the presence of Aa or Pg in healthy individuals may indicate a carrier state in a resistant individual, a factor that is important due to the possible transmission of Aa or Pg among individuals.

It has been stated that cross-sectional data, such as those presented in this study may have a limited value as predictor of future disease activity.[42] However, these data still may be useful to better understand the etiology and progression of ABL in the primary dentition.

Conclusions

1. Spirochetes and motile rods may be part of the indigenous flora of the oral cavity in children. However, their proportions are significantly higher in the subgingival plaque of sites/children with ABL than in sites/children without ABL.
2. In sites where *A. actinomycetemcomitans* was found, higher proportions of spirochetes, lower proportions of cocci, and more CFU of *P. gingivalis* were obtained.
3. Both *A. actinomycetemcomitans* and *P. gingivalis* were found in sites/children without marginal ABL and could not be related to marginal ABL.

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1. Sjödin B, Crossner CG, Unell L, Östlund P: A retrospective radiographic study of alveolar bone loss in the primary dentition in patients with localized juvenile periodontitis. J Clin Periodontol 16:124–27, 1989.
2. Sjödin B, Matsson L, Unell L, Egelberg J: Marginal bone loss in the primary dentition of patients with juvenile periodontitis. J Clin Periodontol 20:32–36, 1993.
3. Shapira L, Smidt A, Van Dyke TE, Barak V, Soskolne AW, Brautbar C, Sela MN, Bimstein E: Sequential manifestation of different forms of early-onset periodontitis: a case report. J Periodontol 65:631–35, 1994.
4. Bimstein E, Delaney JE, Sweeney E: Radiographic assessment of the alveolar bone in children and adolescents. Pediatr Dent 10:199–204, 1988.
5. Bimstein E: Frequency of alveolar bone loss adjacent to proximal caries in the primary molars and healing due to restoration of the teeth. Pediatr Dent 14:30–33, 1992.
6. Bimstein E, Shapira L, Landau E, Sela MN: The relation between alveolar bone loss and proximal caries in children: prevalence and microbiology. ASDC J Dent Child 60:99–103, 1993.
7. Sjödin B, Matsson L: Marginal bone loss in the primary dentition: a survey of 7- to 9-year-old children in Sweden. J Clin Periodontol 21:313–19, 1994.
8. Bimstein E, Treasure ET, Williams SM, Dever J: Alveolar bone loss in 5-year-old New Zealand children: its prevalence and relationship to caries prevalence, socio-economic status and ethnic origin. J Clin Periodontol 21:447–50, 1994.
9. Newman MG, Sanz M, Nisengard R: Host bacteria interactions in periodontal diseases. In: Glickman's Clinical Periodontology, 7th. Ed. Carranza FA Jr, Ed. Philadelphia: WB Saunders, 1990, pp 372–86.
10. Michalowicz BS: Genetic risk factors for the periodontal diseases. Compend Contin Educ Dent 25:1036–48, 1994.
11. Grossi SG, Genco RJ, Machtei EE, Ho AW, Koch G, Dunford R, Zambon JJ, Hausmann E: Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. J Periodontol 66:23–29, 1995.
12. Yoshida-Minami I, Kishimoto K, Suzuki A, Fujiwara T, Shintani S, Morisaki I, Sobue S, Miyamoto M, Nagai A, Kurihara H, Murayama Y, Ooshima T: Clinical microbiological and host defense parameters associated with a case of localized prepubertal periodontitis. J Clin Periodontol 22:56–62, 1995.
13. Syed SA, Loesche WJ: Survival of human dental plaque flora in various transport media. Appl Microbiol 24:638–44, 1972.
14. Listgarten MA, Helldén L: Relative distribution of bacteria at clinically healthy and periodontally diseased sites in humans. J Clin Periodontol 5:115–32, 1978.
15. Mikx FHM, Matee MI, Schaeken MJM: The prevalence of spirochetes in the subgingival microbiota of Tanzanian and Dutch children. J Clin Periodontol 13:289–93, 1986.
16. Loesche WJ, Laughon BE: Role of spirochetes in periodontal disease. In: Host Parasite Interactions in Periodontal Disease. RJ Genco, SE Mergenhagen, Eds. Washington DC: American Society for Microbiology, 1981, pp 62–75.
17. Asikainen S, Alaluusua S, Kleemola-Kujala E: A 2-year follow-up on the clinical and microbial conditions of periodontum in teenagers. J Clin Periodontol 18:16–19, 1991.
18. Jarvis B, Lach VH, Wood JKM: Evaluation of the spiral plate marker for the enumeration of microorganisms in foods. J Appl Bacteriol 43:149–57, 1977
19. Syed SA, Svanberg M, Svanberg G: The predominant cultivable dental plaque flora of beagle dogs in adult gingivitis. J Periodont Res 15:123–36, 1980.
20. Slots J: Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. J Clin Microbiol 15:606–9, 1982.
21. Hunt DE, Jones JV, Dowell VR Jr: Selective media for the isolation of Bacteroides gingivalis. J Clin Microbiol 23:441–45, 1986.
22. Watanabe K: Prepubertal periodontitis: a review of diagnostic criteria, pathogenesis, and differential diagnosis. J Periodont Res 25:31–48, 1990.
23. Blankenstein R, Murray RR, Lindhe J: Prevalence of chronic periodontitis in 13- to 15-year-old children: a radiographic study. J Clin Periodontol 5:285–92, 1978.
24. Källestål C, Matsson L: Marginal bone loss in 16-year-old Swedish adolescents in 1975 and 1988. J Clin Periodontol 18:740–43, 1991.
25. Greenstein G, Polson A, Iker H, Meitner S: Associations between crestal lamina dura and periodontal status. J Periodontol 52:362–66, 1981.
26. Page RC, Schroeder HE: Periodontitis in humans: preva-

lence severity and progression. In: Periodontitis in Man and Other Animals. A Comparative Review. Basel: Karger, 1982, pp 5–17.
27. Benn DK: A review of the reliability of radiographic measurements in estimating alveolar bone changes. J Clin Periodontol 17:14–21, 1990.
28. Bimstein E, Ranly DM, Skjonsby S, Soskolne WA: The effect of facial growth attrition, and age on the distance from the cementoenamel junction to the alveolar bone crest in the primary dentition. Am J Orthod Dentofacial Orthop 103:521–25, 1993.
29. Bimstein E, Garcia-Godoy F: The significance of age, proximal caries, gingival inflammation, probing depths and the loss of lamina dura in the diagnosis of alveolar bone loss in the primary molars. ASDC J Dent Child 61:125–28, 1994.
30. Delaney JE, Ratzan SK, Kornman KS: Subgingival microbiota associated with puberty: studies of pre-, circum-, and postpubertal human females. Pediatr Dent 8:268–75, 1986.
31. Van Winkelhoff AJ, Van Steenbergen TJM, de Graaff J: The role of black pigmented bacteroides in human oral infections. J Clin Periodontol 15:145–55, 1988.
32. Petit MDA, Van Steenbergen TJM, Scholte LMH, Van der Velden U, de Graaff J: Epidemiology and transmission of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* among children and their family members: a report of 4 surveys . J Clin Periodontol 20:641–50, 1993.
33. Van Winkelhoff AJ, de Groot P, Abbas F, de Graaff J: Quantitative aspects of the subgingival distribution of *Actinobacillus actinomycetemcomitans* in a patient with localized juvenile periodontitis. J Clin Periodontol 21:199–202, 1994.
34. Gunsolley JC, Ranney RR, Zambon JJ, Burmeister JA, Schenkein A: *Actinobacillus actinomycetemcomitans* in families afflicted with periodontitis. J Periodontol 61:643–48, 1990.
35. Alaluusua S, Asikainen S: Detection and distribution of *Actinobacillus actinomycetemcomitans* in the primary dentition. J Periodontol 8:504–7, 1988.
36. Frisken KW, Higgings T, Palmer JM: The incidence of periodontophatic microorganisms in young children. Oral Microbiol Immunol 5:43–45, 1990.
37. Wilson ME, Zambon JJ, Suzuki JB, Genco RJ: Generalized juvenile periodontitis, defective neutrophil chemotaxis and Bacteroides gingivalis in a 13-year-old female: a case report. J Periodontol 56:457–63, 1985.
38. Mouton C, Hammond PG, Slots J, Genco RJ: Serum antibodies to Oral Bacteroides asaccharolyticus (Bacteroides gingivalis): relationship to age and periodontal disease. Infect Immun 31:182–92, 1981.
39. Abraham J, Stiles HM, Kammerman LA, Forrester D: Assessing periodontal pathogens in children with varying levels of oral hygiene. ASDC J Dent Child 57:189–93, 1991.
40. Bimstein E, Ebersole JL: Serum antibody levels to oral microorganisms in children and young adults with relation to the severity of gingival disease. Pediatr Dent 13:267–72, 1991.
41. Watson MR, Lopatin DE, Bretz WA Ertel IJ, Loesche WJ: Detection of two anaerobic periodontopathogens in children by means of the BANA and ELISA assays. J Dent Res 70:1052–56, 1991.
42. Griffiths GS, Wilton JMA, Curtis MA, Maiden MFJ, Gillet IR, Wilson DT, Sterne, JAC, Johnson NW: Detection of high-risk groups and individuals for periodontal diseases: clinical assessment of the peridontum. J Clin Periodontol 15:403–10, 1988.