The Relationship Between Gingivitis and Colonization by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in Children

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Background: *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are closely associated with the onset and severity of adult periodontal disease. However, little is known regarding the colonization by, and host antibody response to, these microorganisms in children.

Methods: Plaque and sera were obtained from 40 healthy children, 2 to 18 years old. Gingival health was assessed by the periodontal disease index (PDI), papillary bleeding score (BS) and the modified total papillary margin attachment index (M-PMA). *P. gingivalis* and *A. actinomycetemcomitans* in plaque samples were detected by slot immunoblotting (SIB). Serum antibody levels against these microorganisms were evaluated using ELISA.

Results: More than 60% of the children had detectable levels of *P. gingivalis* in their plaque. Those having detectable levels had more gingival inflammation than those having none; however, these differences were significant only in children over the age of 12 years (PDI, BS). In contrast, while 75% of the children had detectable *A. actinomycetemcomitans,* there were significant differences in gingival inflammation associated with colonization in children from 3 to 7 years of age (PDI) and over 12 years of age (M-PMA). Serum antibody levels to *P. gingivalis* were inversely correlated with gingival inflammation in all age groups, while *A. actinomycetemcomitans* titers were positively correlated with gingival inflammation only in the children over 12 years. No significant relationship between the presence of either *A. actinomycetemcomitans* or *P. gingivalis* and antibodies to them was found.

Conclusions: Our findings show that *P. gingivalis* and *A. actinomycetemcomitans* are readily detected as early as 3 years of age and that their presence is associated with the onset and severity of gingivitis. *J Periodontol 2000;71:403-409.*

KEY WORDS

Gingivitis/microbiology; periodontal diseases/microbiology; antibody formation; *Actinobacillus actinomycetemcomitans*; *Porphyromonas gingivalis*; child.

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Porphyromonas gingivalis and *Actinobacillus actinomycetemcomitans* are closely associated with periodontal disease.[1-5] These 2 putative periodontal pathogens have been shown to produce disease in animal models,[6-8] and are commonly isolated from destructive periodontal lesions in both juveniles and adults.[9] *P. gingivalis* has been reported to be a dominant pathogen in adult periodontitis.[3] *A. actinomycetemcomitans* was originally characterized as a major etiologic agent in juvenile forms of periodontitis in addition to adult periodontitis.[2,7,10]

Several earlier investigations sought causative factors leading to gingivitis and periodontitis in children. Savitt and Kent[11] reported that *A. actinomycetemcomitans* was present in the subgingival plaque of older children (10 to 19 years). Alaluusua and Asikainen[12] found that *A. actinomycetemcomitans* was frequently a member of the oral flora of children with a primary dentition. Barr-Agholme et al.[13] reported that *A. actinomycetemcomitans* contributed to the development of gingival inflammation in younger children with Down's syndrome and that *P. gingivalis* was most strongly associated with the severity of pubertal periodontitis. There are, however, few studies that examine microbial colonization and gingival health across the entire span of childhood in healthy populations.[14] Thus, the objective of our study was to evaluate the relationship between the

detection of *P. gingivalis* and *A. actinomycetemcomitans* in the subgingival plaque, the presence of serum antibodies to these microorganisms, and the level of gingival inflammation in a population of healthy children aged 2 to 18 years.

MATERIALS AND METHODS

Study Population

All protocols involving human subjects were approved by the Institutional Review Committee of Kagoshima University Dental School, and the study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1983. Forty children of varied home backgrounds, aged 2 to 18 years, were recruited as study subjects from patients in the Kagoshima University Dental School Pediatric Clinic. The selection criteria were that they possessed good general health and met the age-group requirement. As shown in Table 1, the subjects were stratified into 4 equal (n = 10) age groups (G1, under 3 years; G2, 3 to 6 years; G3, 7 to 11 years; and G4, over 12 years.) None of the subjects was undergoing treatment for systemic or oral diseases or other conditions that would be expected to contribute to the alteration of their microbial flora or immune response. Approximately 25% of the samples obtained were from sites without clinical indication of gingivitis.

Gingival Health

The extent of gingival inflammation was assessed by using the modified total papillary margin attachment index (M-PMA) of all sites based on the PMA index by Massler,[15] papillary bleeding score (BS) of Loesche,[16] and periodontal disease index (PDI) by Ramfjord.[17] Examinations were performed by the same operator.

Subgingival Plaque Specimens

After removal of both marginal and supragingival plaque, subgingival plaque was collected from the sulcus of the buccal site and buccal portions of the interdental crevice of an upper molar with a sterile #6 dental explorer.[18] This site was selected because it is an area susceptible to gingivitis and is easily sampled in children. Plaque samples were placed in sterile cryotubes containing 0.5 ml of phosphate-buffered saline (PBS; 0.05M sodium phosphate, 0.15M NaCl, [pH 7.5]) containing 0.5% formaldehyde, EDTA (2.0 mM), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A (0.1 mM), and leupeptin (0.5 mg/l) and stored at –20°C until assayed.

Table 1.

Age Categories

Age Group (Years)	Category	Male	Female	Average Age (Months)	Total
<3	G1	7	3	28.5	10
3 to <7	G2	6	4	54.0	10
7 to <12	G3	5	5	112.2	10
>12	G4	5	5	173.4	10
Totals		23	17	92.0	40

Serum Specimens

Five ml of blood was collected from the saphenous vein without anticoagulants. After clotting and centrifugation, sera were stored frozen at –70°C until assayed.

Antigens

Pure cultures of *P. gingivalis* (ATCC 33277) and *A. actinomycetemcomitans* (ATCC 43718) were used as antigens in ELISA. These microorganisms were grown in Schaedler broth supplemented with 5 µg of hemin and 1 mg of menadione per ml under anaerobic conditions (85% nitrogen, 10% hydrogen, and 5% carbon dioxide) at 37°C. The microorganisms were harvested by centrifugation (13,000 × g) at late log phase in the presence of protease inhibitors (2 mM EDTA, 0.5 mM PMSF; 10^{-7} M pepstatin A, 0.5 mg/ml leupeptin). The cell pellets were washed 3 times with PBS containing 1 mM EDTA and then fixed by suspension in PBS containing 0.5% formalin for 16 to 18 hours. The formalinized bacteria were washed three more times with PBS solution containing 1 mM EDTA, then resuspended in distilled water, lyophilized, and stored at –20°C.

Antibacterial Antibodies

High-titer (>1:40,000 as determined by ELISA) polyclonal antibodies against *P. gingivalis* and *A. actinomycetemcomitans* were produced in rabbits. Briefly, the rabbits were immunized subcutaneously with 1 mg (dry weight) of the bacterial preparation suspended in 1 ml of aluminum hydroxide adjuvant gel. The rabbits were reimmunized after 1 week and boosted approximately 5 weeks following the first immunization. Bleedings were obtained weekly after the booster immunization. All antisera were screened by the micro-ELISA procedure for titers and specificity to both related and unrelated species. The antisera were screened against approximately 70 American Type Culture Collection strains and clinical isolates representing 10 genera and 25 species. The antisera reacted strongly with all isolates determined to be of the same species as that used for immunization. The anti-*A. actinomycetemcomitans* was shown to react with all major serotypes of *A. actinomycetemcomitans*. Pre-immune rabbit sera provided a control for non-specific binding to the antigen. At the final dilutions used in the assays, these antisera were specific for the immunizing antigens.

Antibody Measurements

Antibacterial antibody levels in the sera of subjects were measured using the micro-ELISA procedure and were expressed as an ELISA rate (absorbance units/minute). This method of antibody measurement was previously reported by Morinushi et al.[19] Briefly, formalinized microorganisms were diluted in 0.1 *M* sodium carbonate coating buffer to a final concentration of 1.0 to 10.0 µg/ml and dispensed (0.1 ml/well) into 96-well microtiter plates and incubated for 2 hours at 37°C. The plates were then stored at 4°C until used. Before use, the sensitized plates were washed with PBS containing 0.05% Tween-20 (PBS-T).

Serial dilutions of patient sera in PBS-T, starting at 1:100, were added to the plates and incubated for 1 hour at 37°C. After washing with PBS-T, 0.1 ml (1:1,000 dilution) of γ-chain-specific goat anti-human immunoglobulin conjugated to alkaline phosphatase,[‡] was added to each well and incubated for 1 hour at 37°C. After a final wash with PBS-T, 0.1 ml alkaline phosphatase substrate[§] (1 mg/ml) was added to each well, and the A_{405} of each well was measured at 10-minute intervals using a multichannel spectrometer. A reference serum composed of a pool of at least 20 healthy adult sera, possessing antibody against both microorganisms, was also titrated in each plate to allow internal standardization. Control wells, containing the antigen and conjugate, were included to detect any background attributed to non-specific binding of the conjugate. The ELISA rates were calculated as the rate of absorbance change per minute, which does not depend on development to an arbitrary endpoint.

Microbial Detection

Detection and quantitation of the 2 microorganisms were done by the slot immunoblot assay (SIB).[20] Prior to the assay, the plaque samples were subjected to ultrasonication[‖] (3 to 5 seconds; 60% power) for disruption of any plaque aggregates.

Nitrocellulose membranes[¶] (BA-85) were soaked for 15 minutes in TBS (0.05 *M* NaCl, 10.0 mM Tris, pH 7.4) prior to insertion in the slot blot manifold.[#] Microbial standards (pure cultures) or undiluted plaque samples were applied (10 µl) to each well of the slot blot manifold, which was then evacuated with the gentle application of vacuum. The nitrocellulose membranes were removed from the manifold for all further processing. Subsequent washes and incubations were performed in glass dishes with gentle rocking at room temperature.

The nitrocellulose membranes were next immersed in TBS containing 0.5% non-fat dried milk[21] for 30 to 60 minutes to block unoccupied binding sites on the nitrocellulose membrane. The appropriate antibacterial polyclonal rabbit antibody, diluted (1:1,000) in TBS-Tween (TBS-T) containing 0.5% milk, was applied and allowed to incubate for 1 hour. Following three 5-minute washes in TBS-T, the membranes were immersed for 1 hour in goat anti-rabbit IgG conjugated to alkaline phosphatase,[**] diluted 1:1,000 in TBS-T containing 0.5% milk. After three 5-minute washes, the membranes were immersed in BCIP-NBT substrate solution[††] until color development was completed. Prior to microbial analysis, detection of gross carbohydrate and protein levels of each plaque specimen was performed to verify that sufficient plaque was present to render a meaningful estimation of specific microbial presence.[21] Semiquantitation of bacterial presence was determined by visually scoring the color intensity of the bands on each membrane. A score of 0 was assigned to slots where no band was detected. A score of 1 to 3 was assigned to detectable bands, which corresponds to at least 2×10^4 bacteria per sample.

Table 2.

Incidence of Gingivitis at Sampling Site

N	G1	G2	G3	G4
With gingivitis	5	9	10	5
Without gingivitis	5	1	0	5
Totals	10	10	10	10

Statistical Analysis

The 40 subjects were each assigned to 1 of 4 groups: G1 (pre-deciduous dentition), G2 (deciduous dentition), G3 (mainly mixed dentition), and G4 (permanent dentition). There were 10 subjects in each group. The relationship between the gingivitis indices, antibody levels, and microbial detection, by age group, was analyzed by ANOVA for parametric measurement. The Spearman rank correlation coefficient and Kruskal-Wallace tests were used for non-parametric determinations. Analyses of differences between categories were performed by *t* tests.

RESULTS

Samples

Table 2 displays the distribution of samples obtained from healthy or inflamed sites by age group. It can be seen that groups G2 and G3 had the highest incidence of inflammation, comprising 65% of all inflamed sites and 95% of sites within the 2 groups. Of the 40 teeth from which plaque samples were taken, 11 exhibited no clinical signs of gingivitis.

‡ Zymed, South San Francisco, CA.
§ Sigma Chemical Co., St. Louis, MO.
‖ Kontes Instruments, Vineland, NJ.
¶ Schleicher & Schuell, Keene, NH.
# Minifold II, Schleicher & Schuell.
** Bio-Rad Laboratories, Hercules, CA.
†† Kirkegaard & Perry Laboratories, Gaithersburg, MD.

Table 3.

Gingival Status Stratified by Age (n = 10 each group)

Index	G1 Mean ± SD	G2 Mean ± SD	G3 Mean ± SD	G4 Mean ± SD
PDI	0.47 ± 0.45	1.09 ± 0.56	1.15 ± 0.44	0.72 ± 0.54
BS	0.62 ± 0.64	1.14 ± 0.76	1.91 ± 0.57	1.33 ± 0.79
M-PMA	0.19 ± 0.19	0.24 ± 0.13	0.36 ± 0.14	0.23 ± 0.15

* Arrows indicate significant differences between specific cells ($P<0.05$).

Table 4.

Relationship Between Gingival Status and *P. gingivalis* Colonization

Gingival Score/ Group	SIB Score ≥1 Mean ± SD	SIB Score 0 Mean ± SD	Significance (*P* Value)
PDI	1.01 ± 0.52 (n = 25)	0.61 ± 0.55 (n = 15)	**<0.05**
G1	0.62 ± 0.45 (n = 7)	0.11 ± 0.19 (n = 3)	ns
G2	1.03 ± 0.69 (n = 6)	1.21 ± 0.35 (n = 4)	ns
G3	1.23 ± 0.43 (n = 8)	0.85 ± 0.49 (n = 2)	ns
G4	1.21 ± 0.16 (n = 4)	0.39 ± 0.43 (n = 6)	**<0.01**
BS	1.46 ± 0.78 (n = 25)	0.89 ± 0.78 (n = 25)	**<0.03**
G1	0.86 ± 0.62 (n = 7)	0.06 ± 0.09 (n = 3)	ns
G2	1.09 ± 0.56 (n = 6)	1.22 ± 1.08 (n = 4)	ns
G3	2.04 ± 0.56 (n = 8)	1.38 ± 0.18 (n = 2)	ns
G4	1.94 ± 0.66 (n = 4)	0.92 ± 0.61 (n = 6)	**<0.04**
M-PMA	0.30 ± 0.16 (n = 25)	0.18 ± 0.14 (n = 25)	**<0.03**
G1	0.25 ± 0.20 (n = 7)	0.03 ± 0.05 (n = 3)	ns
G2	0.27 ± 0.16 (n = 6)	0.19 ± 0.05 (n = 4)	ns
G3	0.36 ± 0.15 (n = 8)	0.36 ± 0.14 (n = 2)	ns
G4	0.32 ± 0.11 (n = 4)	0.18 ± 0.15 (n = 6)	ns

Bold type indicates statistical significance.

Clinical Indices

Table 3 shows the relationship between age and mean gingival inflammation scores. As shown, irrespective of the clinical index used, there is a progressive increase in gingival inflammation through age category G3 (<12 years). Likewise, by all measures used, inflammation diminishes in the G4 group (>12 years).

Table 5.

Relationship Between Gingival Status and *A. actinomycetemcomitans* Colonization

Gingival Score/ Group	SIB Score ≥1 Mean ± SD	SIB Score 0 Mean ± SD	Significance (*P* Value)
PDI	0.91 ± 0.59 (n = 30)	0.72 ± 0.45 (n = 10)	ns
G1	0.47 ± 0.45 (n = 10)	0	—
G2	1.47 ± 0.13 (n = 6)	0.54 ± 0.46 (n = 4)	**<0.01**
G3	1.23 ± 0.43 (n = 8)	0.85 ± 0.49 (n = 2)	ns
G4	0.64 ± 0.60 (n = 6)	0.83 ± 0.49 (n = 4)	ns
BS*	1.27 ± 0.89 (n = 30)	1.19 ± 0.59 (n = 10)	ns
M-PMA	0.25 ± 0.17 (n = 30)	0.28 ± 0.13 (n = 10)	ns
G1	0.19 ± 0.20 (n = 10)	0	—
G2	0.29 ± 0.13 (n = 6)	0.17 ± 0.10 (n = 4)	ns
G3	0.36 ± 0.15 (n = 8)	0.36 ± 0.14 (n = 2)	ns
G4	0.16 ± 0.13 (n = 6)	0.34 ± 0.11 (n = 4)	**<0.05**

* No age groups within this category had statistically significant differences.
Bold type indicates statistical significance.

Bacterial Colonization and Clinical Measurements

As shown in Table 4, more than 60% (25) of the children had detectable levels of *P. gingivalis* in their plaque. When evaluated as a single group, the children having detectable levels of *P. gingivalis* had significantly higher gingival inflammation scores than did children with no detectable *P. gingivalis* in their plaque. When assessed by individual age categories, these differences were statistically significant in the G4 age group as evaluated by the PDI and BS for signs of gingival inflammation.

In contrast to the above are the findings for *A. actinomycetemcomitans,* shown in Table 5. When assessed as a single group, fully 75% (30) of the children showed evidence of detectable colonization by *A. actinomycetemcomitans,* yet the mean gingival inflammation scores were not statistically significant from those of children without detectable levels of *A. actinomycetemcomitans*. When individual age categories were evaluated, the PDI score was found to be significantly higher in colonized children at G2. No significant differences were identified using the BS measurements. When assessed by the M-PMA, children in the G4 age group who were colonized by *A. actinomycetemcomitans* had a significantly lower level of gingival inflammation.

Table 6 compares the colonization data obtained from inflamed or non-inflamed sites. It can be seen

Table 6.

Bacterial Detection and Site Status

Bacterium	Detected	Not Detected	Total
P. gingivalis			
Non-inflamed site	4	7	11
Inflamed site	21	8	29
Total	25	15	40
A. actinomycetemcomitans			
Non-inflamed site	9	2	11
Inflamed site	21	8	29
Total	30	10	40

that, in our sampled population, *P. gingivalis* and *A. actinomycetemcomitans* were equally likely to be found in about 70% of inflamed sites. On the other hand, when comparing detection rates in non-inflamed sites, *A. actinomycetemcomitans* was detected in 82% of the sites while *P. gingivalis* was found in only 36% of healthy sites.

As seen in Table 7, if one compares mean colonization scores in the 4 age categories, there is evidence of an overall trend to decreasing colonization with increasing age for both *A. actinomycetemcomitans* and *P. gingivalis*. However, no statistically significant differences in colonization for either microorganism were detected between age groups.

Antibody Titers and Clinical Measurements

Finally, we examined the relationships between the three clinical indices and the serum antibody levels to *P. gingivalis* and *A. actinomycetemcomitans*. *P. gingivalis* serum antibody measurements were found to be inversely correlated with increased PDI and M-PMA scores (P <0.03, r = –0.6979) irrespective of age. Among children in the G4 age group, *A. actinomycetemcomitans* serum antibody levels were significantly correlated with the PDI score (P <0.03, r = 0.7044). However, for both microorganisms, there was no significant difference between titers when analyzed by ANOVA. When relationships between antibody titer and colonization by either *P. gingivalis* or *A. actinomycetemcomitans* were examined, no statistically significant associations were discerned.

Table 7.

Colonization by *P. gingivalis* and *A. actinomycetemcomitans* by Age

	G1 Mean ± SD	G2 Mean ± SD	G3 Mean ± SD	G4 Mean ± SD
*P. gingivalis**	1.40 ± 0.52	0.80 ± 0.79	0.80 ± 0.42	0.60 ± 0.52
*A. actinomycetemcomitans**	1.00 ± 0.82	1.09 ± 0.34	0.80 ± 0.42	0.60 ± 0.84

* No statistically significant differences between age categories by ANOVA.

DISCUSSION

If childhood gingivitis could be monitored by the detection of key indicator microflora and ultimately be shown to be related to the onset and severity of adult periodontitis, it would have considerable impact on prevention. The stage could then be set for earlier intervention and, possibly, modulation or elimination of periodontal disease in adults. Based on data obtained from animal models, Saxe et al.[22] and Lindhe et al.[23] have advanced the proposition that gingivitis may represent a pre-phase to periodontitis. *P. gingivalis* and *A. actinomycetemcomitans* are 2 microorganisms closely associated with the onset and severity of periodontal disease.[1-5] Reports on colonization by, and antibody response to, these 2 microorganisms in childhood are few and fewer still in children under 4 years old.[14,24] In view of the paucity of data concerned with childhood periodontal pathogenesis and its role in the continuum to adult disease states, further investigation and discussion seem warranted.

This study was undertaken to examine, both directly and indirectly, the presence of *P. gingivalis* and *A. actinomycetemcomitans* in the oral microflora of young children and their association with clinical gingival pathology. *P. gingivalis* was detected in the subgingival plaque of over 60% of the children enrolled for this program, and *A. actinomycetemcomitans* was present in 75% of the samples. These organisms were detected with comparatively high incidences in subgingival plaque from children under the age of 3 years (G1).

In a related study, Mouton et al.,[14] looking at *Bacteroides asaccharolyticus (B. asaccharolyticus)* in children 6 months to 12 years of age and controlled for transplacental transfer of maternal antibodies, demonstrated the presence of IgG antibodies reactive with *B. asaccharolyticus*. Further, they pointed out that the primary locus for recovery of oral *B. asaccharolyticus* was the gingival sulcus, and that the development of the gingival sulcus is the primary requisite for the establishment of *B. asaccharolyticus*. Their conclusions included the possibility of colonization by *B. asaccharolyticus* in children over 2 years old whose primary teeth are erupting. Nakagawa et al.,[24] looking at *A. actinomycetemcomitans,* reported that the distribution of patients found to have had positive plaque samples was 20% in a population whose mean age was 4.5 years.

Alaluusua and Asikainen[12] indicated that *A. actinomycetemcomitans* was a rather frequent constituent of the oral flora in samples obtained from the mesial surface of primary molars and sulcular sampling of the proximal contact of the primary molar. These sites are similar to those used in our study for obtaining plaque from the primary teeth of children 4 to 7 years old. Alaluusua and Asikainen[12] also observed that the primary molar and the dorsum of the tongue seemed to be favored sites for *A. actinomycetemcomitans*. Our finding that *P. gingivalis* and *A. actinomycetemcomitans* are prevalent in subgingival plaque from children less than 3 years of age is consistent with these reports. What is novel in this study is the finding of a significant relationship between the presence of *P. gingivalis* and clinical diagnosis of gingivitis in these young children ($P<0.02$). In addition, although not approaching statistical significance, the relationship between detectable *A. actinomycetemcomitans* in plaque and the clinical values for gingivitis was of interest.

Mombelli et al.[25] reported a high incidence of isolating *P. gingivalis* from deep molar pockets which bled upon probing. Slots and Listgarten[4] showed that *P. gingivalis* levels were elevated in the plaque collected from actively bleeding sites. Savitt and Kent[11] reported that they found *A. actinomycetemcomitans* to be prevalent in their youngest age group (10 to 19 years) but that it decreased in prevalence with age. *P. gingivalis,* on the other hand, was much more closely associated with older subjects (30 years and older). Nakagawa et al.[24] showed that young gingivitis patients with positive plaque samples for *A. actinomycetemcomitans* comprised 20% of preschool age children (mean age 4.5 years) and that those numbers declined to 10% for early school age children (mean age 9.1 years). These reports, which are consistent with our own, demonstrate the close association between colonization by *A. actinomycetemcomitans* and the onset of gingivitis in early childhood (3 to 7 years) and its severity in adolescence. *P. gingivalis* is associated with the progression of gingivitis and the onset of periodontitis in adolescence (12 years and older).

Although we observed statistically significant relationships between gingivitis and antibody titers, we did not find any direct relationships between colonization and titers. This is also consistent with our previous reports.[19] The same explanations are offered: 1) sensitization to certain microbial antigens is the result of chronic low-level exposure and not a reflection of rapid acute changes; 2) sensitization to specific plaque microorganisms is the result of short, acute episodes of exposure; 3) antibodies are actually the result of stimulation from cross-reactive antigens which are unrelated to the oral status of patients; and 4) the lack of direct correlation between microbiologic analysis and immunity may be tied to an innate load-lag relationship between the two, especially if the presence of the microorganism is episodic. Further studies will be required to establish the actual mechanism.

In conclusion, our findings show that *P. gingivalis* and *A. actinomycetemcomitans* are readily detected in healthy young children (2 to 3 years old), and that *A. actinomycetemcomitans* and *P. gingivalis* are closely related to the onset and severity of gingivitis in healthy children above the age of 3 years. The presence of *P. gingivalis* is most strongly associated with the progression of gingivitis and onset of periodontitis in healthy children. Clearly, there is no direct proof that pediatric colonization by *P. gingivalis* or *A. actinomycetemcomitans* predisposes an individual to adult periodontitis. However, the earlier their infection is established, the greater the opportunity the microorganisms have to establish themselves in that ecological niche. Additional longitudinal studies will be required to establish this relationship.

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