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Can salivary activity predict periodontal breakdown in *A. actinomycetemcomitans* infected adolescents?

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Abstract

Objective—While *Aggregatibacter actinomycetemcomitans* (*Aa*) is highly associated with localized aggressive periodontitis (LAP) many *Aa*-carriers do not develop LAP. This study was designed to determine whether specific salivary factors could distinguish between subjects who have *Aa* initially and remain healthy (H/AA) as compared to those who develop LAP (LAP/AA).

Design—H/AA subjects and healthy controls with no *Aa* (H) were enrolled in a longitudinal cohort study to investigate initiation of bone loss (LAP) over 3 years. After detection of LAP, stored saliva from 10 H, 10 H/AA, and 10 LAP/AA subjects was thawed, processed, and tested for; 1) lactoferrin (Lf) concentration and iron levels, 2) agglutination of *Aa*; 3) killing of Gram-positive bacteria.

Results—LAP/AA saliva levels of Lf iron were low prior to and after bone loss (3.6 ± 1.7 ng Fe/ μ g (LAP/AA vs. H and H/AA $p = 0.01$). Saliva from H/AA subjects caused *Aa* to agglutinate significantly more than H or LAP/AA saliva ($p = 0.01$). LAP/AA saliva killed *Streptococcus mutans*, *S. sanguis* and *Lactobacillus* in vitro by $> 83\%$. Saliva from H individuals killed these bacteria by $< 3.3\%$ (LAP/AA vs. H; $p = 0.01$). H/AA killing was intermediate.

Conclusions—LAP/AA saliva showed: low levels of Lf iron, minimal *Aa* agglutinating activity, and high killing activity against Gram-positive bacteria. *Aa*-positive healthy saliva (H/AA) showed: higher levels of Lf iron, maximal *Aa* agglutinating activity, and moderate killing of Gram-positive bacteria. A salivary activity profile can distinguish between subjects who are *Aa*-positive and remain healthy from those who develop LAP.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Keywords

Periodontics; Localized aggressive periodontitis; Microbiology; *Aggregatibacter actinomycetemcomitans*; Salivary activity; Lactoferrin; agglutination; killing gram-positive bacteria

Introduction

Saliva bathes all oral and dentate surfaces with numerous bioactive substances.¹ While the role of saliva has been extensively studied in relationship to caries, considerably less emphasis has been placed on the role of saliva in periodontal disease.² Our group has been studying *Aggregatibacter actinomycetemcomitans* induced localized aggressive periodontitis (LAP) in a longitudinal model that follows African-American and Hispanic adolescents at 6 month intervals for a period of 2–3 years.³ Subjects enrolled in the study were periodontally healthy and were either *Aa*-positive or *Aa*-negative at screening.³

In agreement with the majority of microbiological research published our study indicated that *Aa* was highly associated with LAP in children of African descent.^{4,5} Moreover, all subjects in our study who developed bone loss within the 2–3 year period had *Aa* at screening. However, we also found that up to 70% of those who had *Aa* at screening remained healthy.³ Thus, as seen in other studies, a significant percentage of *Aa*-carriers did not develop detectable LAP.⁶ The objective of the current research is to determine whether a profile of specific salivary factors can distinguish between subjects who have *Aa* initially and remain healthy as compared to those subjects who have *Aa* initially and go on to develop LAP.

The salivary factors that we chose to study included, salivary lactoferrin, saliva induced agglutination and salivary killing. Salivary lactoferrin (Lf) with high levels of iron has been shown to interfere with binding of oral microorganisms to buccal epithelial cells (BECs); while Lf with low iron does not affect binding in-vitro.^{7,8} Thus *Aa*-carriers with low levels of Lf iron could retain *Aa* on their mucosa and thus be more susceptible to *Aa*-induced LAP.⁹

Saliva, particularly salivary IgA, can cause oral bacteria to agglutinate and form large aggregate clumps. Salivary agglutination could facilitate removal of these bacteria from dentate surfaces.^{10–12} Thus *Aa*-carriers with saliva with minimal agglutinating activity against *Aa* would be more likely to retain *Aa* and thus those individuals would be more susceptible to *Aa*-induced LAP.

Factors in saliva have been shown to modulate the growth of pioneer colonizing bacteria and thus could influence bacterial ecology which could affect *Aa* distribution in the plaque biofilm.^{13,14} Thus *Aa*-carriers who have saliva that kills Gram-positive plaque bacteria could provide *Aa*-carriers with a competitive advantage that would favor growth and survival of *Aa* on tooth and tissue surfaces. Subjects with saliva that kills competing bacteria could be more likely to retain *Aa* and those individuals would be more susceptible to *Aa*-induced LAP.

In light of the fact that a high percentage of *Aa*-carriers do not develop LAP^{3,6} our research goal is to determine if we could identify salivary factors that, singly or in combination, could provide *Aa* with a favorable environment for survival. Salivary factors that favor *Aa* colonization, growth and survival could distinguish between subjects who have *Aa* initially and remain healthy from those who have *Aa* and go on to develop LAP. A salivary assay that makes this distinction could provide clinicians with a way of identifying subjects who will succumb to *Aa*-induced LAP in the future.

2. Materials and methods

2.1 Study Design

The study was conducted over a three-year period from 2008–2011. Prior to the start, approval was obtained from the Institutional Review Board of the University of Medicine and Dentistry of New Jersey and the review boards of the Elementary, Middle School and from the local High School Systems in Newark, New Jersey. Signed consent from the subjects' parents or guardians as well as the subjects' oral consent was attained to enable participation in the study. Subjects, ages 12–17, who were medically healthy were then evaluated for caries and periodontal disease, sampled for *Aa*, and entered into the longitudinal study.³

The current study utilizes stored salivary samples obtained from a subset of patients who participated in a longitudinal cohort study of LAP in adolescents previously published.³ In that study subjects were screened and classified as either *Aa*-positive or negative, enrolled, recalled at 6-month intervals for 3 years to assess the relationship between *Aa* carriage and LAP initiation.³ In this report (Table 1) we examine ten *Aa*-positive subjects who developed bone loss (defined as LAP/AA), ten subjects who were *Aa*-positive who remained healthy (H/AA) and ten *Aa*-negative subjects who remained healthy (H). In the longitudinal study, clinical measurements were performed and samples were collected and stored over the 3-year period. Retrospectively, after disease occurred the stored samples from those subjects and from matched subjects who remained healthy were processed to evaluate specific salivary elements within these samples that could be associated with initiation of LAP.³

To date approximately 2,500 subjects have been screened. Our subject population of thirty in this study was drawn from the 175 subjects who were followed for two years or more. In addition to the 30 matched subjects examined in this study, saliva from three additional subjects who had LAP at screening was used in the Lf assays exclusively since only a limited amount of saliva from these participants was available.

2.2 Clinical protocol: screening visit and recall

The purpose of the screening visit was to determine the level of disease and whether the student was a carrier of *Aa*. Briefly, sampling was performed and subjects were examined for oral soft tissue lesions, caries, and periodontal disease and then rescheduled for recall.

The purpose of the recall visit was to determine if the subject had developed periodontal disease and if so whether he or she was *Aa*-positive or negative. At the recall visit two to four horizontal bitewing radiographs were taken for the detection of early bone loss.³ Since

we were studying LAP we focused on the molars and incisors to diagnose disease by radiograph.

2.3 Clinical periodontal measurements

Probing depths and attachment levels were recorded and considered; however, evidence of bone loss was required to define LAP.³ Examiners were calibrated and blinded as described previously.³ When bone loss was detected, the subject was exited from the study and referred for treatment at the university's practice facility. If no bone loss was detected subjects were returned to the 6-month recall schedule for up to 3 years.³

2.4 Saliva collections and testing

Four to five milliliters of saliva was obtained from each of the subjects and divided into three aliquots in order to test for; 1) salivary Lf concentration and iron levels, 2) sIgA agglutination, and 3) salivary killing of Gram-positive bacteria. For saliva collection, subjects were given three minutes to expectorate into a 50-ml wide-mouthed polystyrene tube placed over ice. Saliva was stored at -80°C for future testing. After thawing, samples were clarified by centrifugation in preparation for testing from subjects in the three groups; periodontally healthy/*Aa*-positive subjects (H/AA), periodontally healthy/*Aa*-negative subjects (H) and LAP *Aa*-positive subjects (LAP/AA).

2.5 Determination of Lf concentration and iron content in clarified saliva

Lf concentration was determined using the protocol for Hycult biotechnology Lactoferrin ELISA kit (Plymouth Meeting PA, USA). As such Lf standards were prepared to achieve a range from 0.4 to 100 ng/ml of Lf. 100 μl in duplicate was transferred from each standard into the wells of the ELISA tray. The test samples of saliva from the subjects was thawed 30 minutes prior to analysis and then clarified via centrifugation at 11,000 rpm for 10 minutes. 20 μl of the supernatant was then placed into a new microcentrifuge tube to which was added 180 μl of standard dilution buffer. 100 μl (in duplicate) from each of the prepared saliva samples was placed in the wells. The tray was covered and incubated at 37°C and the standard Hycult protocol was followed. The reaction was stopped by addition of 100 μl of 2.0 M citric acid and then read by a spectrophotometer at 450 nm. To determine Lf concentration for salivary samples, calculations were based on fitting the sample reading to the standard curve derived from the Lf standards.

Determination of iron content per μg of Lf protein in whole saliva was performed using "The QuantiChrom Iron Assay Kit" from BioAssay Systems (Haywood, CA, USA). The assay was modified for this analysis by first using the Hycult Lf assessment protocol as described above and then extracting Lf iron for analysis. To start, reagents were admixed as described in the QuantiChrom iron assay. The iron standards supplied in the kit were diluted and used to construct a standard curve to assess levels of iron. Following this preparative step, 100 μl of saliva from each of the samples was then placed into one well of a 96 well Hycult plate designed to capture Lf as described above. 100 μl of stop solution was added and the plates were allowed to stand for 30 minutes at room temperature. Following this 25 μl was transferred into a second well. After this 75 μl of 0.1 N of NaOH was added to neutralize the mixture. 50 μl of this volume was transferred into a third well of the 96 well

plate used to assess iron content as described below. 200 µl of the prepared Iron Assay Working Reagent was added to each well of this third 96 well plate. The plates were incubated for 40 minutes at room temperature and then read in a spectrophotometer at 590 nm. To calculate iron concentration of the Lf found in the saliva, absorbance of each sample was compared to levels derived from the standard curve.

2.6 Salivary agglutination protocol

Saliva used in these assessments had a total salivary sIgA concentration that ranged from 200 to 290 µg/ml of protein. Total protein was also estimated using a biuret assay.¹⁵ In the first assay a smooth, non-autoaggregating *Aa* strain, JP2, was cultured in AAGM broth¹⁶ at 37°C in 10% CO₂ for 1 day and then adjusted in PBS to an A₅₉₀ = 0.8 (approximately 4.4 × 10⁸ Colony Forming Units (CFU)/ml). This stock culture was serially diluted and plated on AAGM agar to determine the live JP2 in CFU/ml.¹⁶ One hundred µl of either clarified saliva from each subject or PBS (control) was admixed with 100 µl of adjusted culture of *Aa* strain JP2.¹⁷ Microfuge tubes were incubated for 5 minutes at 37°C. 100 µl from each tube was pipetted into a 15 ml centrifuge tube containing 10 ml of 7.0 % Ficoll 400. The samples were then centrifuged at 1,000×g to pellet the aggregates and separate them from single cells. The Ficoll was decanted and the pellets re-suspended in 100 µl of sterile PBS. Each sample was then subjected to vortex agitation for 10 seconds to breakup the aggregates. The re-suspended sample was serially diluted in sterile PBS, and then aliquots were plated on AAGM agar. Plates were incubated overnight at 37°C in 10% CO₂ and then counted to determine CFU/ml of aggregated bacteria. The level of aggregation or agglutination was calculated by dividing the CFU/ml of aggregated bacteria by the starting concentration of bacteria mixed with saliva or PBS. Since the JP2 strain showed minimal aggregation the aggregates formed in saliva were compared to the aggregates of the starting dose to determine the percentage of agglutination caused by interaction with saliva.

In the second assessment we determined the percentage of aggregation normalized to the concentration of sIgA per unit of saliva. Human salivary samples were added to ELISA plates containing polyclonal anti-human IgA made in rabbits (ALPCo, Salem, NH USA). After incubation salivary IgA now captured on the ELISA plate was washed and a secondary antibody to human secretory IgA made in mice and conjugated to peroxidase was then added to determine the level of salivary IgA in the salivary sample. Once this was determined the percentage of agglutination per unit of sIgA was calculated.

The third assessment was used to determine whether the main aggregating factor in saliva was IgA. To do this 200 µl of either Protein A Agarose Slurry (Thermo Scientific, Rockford, IL) or Protein L Agarose Slurry (Thermo Scientific, Rockford, IL) were pipetted into a microfuge tube and centrifuged at 2,500 × g for 3 minutes. The agarose pellet was then washed 3 times with sterile PBS, pH 7.4. The pellet was then mixed with 100 µl of clarified saliva and incubated for 2 hrs at 37°C rotating at 30 rpm /min. The admixed sample was then centrifuged at 2,500×g for 3 minutes to pellet Protein A or Protein L Agarose and the treated saliva was decanted and saved for the agglutination assay which was performed as described above. The percentage of agglutinating cells in the Protein L or Protein A pre-treated saliva was calculated as previously described.

2.7 Salivary/ bacterial killing assessment

Experiments consisted of taking clarified saliva from each subject in each of the three groups as described above. 50 µl of overnight adjusted stock cultures ($A_{590}=0.8$) of *S. sanguis* G9B, *S. mutans* 25175 and a clinical *Lactobacillus* species were pipetted into microfuge tubes along with 50 µl of clarified saliva. The tube was subjected to vortex agitation for 10 seconds. The microfuge tubes containing the bacteria and saliva were incubated at 37°C. The samples were rotated at 25 revolutions per minute for 2 hrs. The microfuge tubes were then centrifuged at 11,000×g for 30 seconds to pellet the bacteria and the supernatant was decanted. The pellets were re-suspended in 100 µl of sterile PBS. Each tube was serially diluted in PBS and plated on the agar appropriate for each bacterium (Tomato Juice agar, Fisher Scientific, Pittsburgh PA, USA) for lactobacilli, Mitis-Salivarius agar (Fisher Scientific) for *S. sanguis*, and Mitis-Salivarius agar plus bacitracin and sucrose (Fisher Scientific) for *S. mutans*. All plates were incubated aerobically at 37°C overnight and counted to determine the CFU/ml. Controls consisted of aliquots to which 50 µl of PBS was added following the same protocol. Protein content for the saliva was calculated¹⁵ for each individual and results were normalized to reflect the CFU/ml/µg of protein and compared to growth in the PBS control solution.

2.8 Data Analysis

The salivary activity profile that we presumed would be congruent with *Aa*-survival was saliva containing; 1) lowered Lf iron concentration, 2) lowered IgA agglutinating ability, and 3) elevated ability kill competing pioneer colonizing Gram-positive bacteria.

Lf iron concentrations per ng of Lf were found to be 4-fold lower in LAP subjects as compared to controls in a previous study.⁹ Calculations derived from this data indicated that a sample size of 7 was needed to obtain power of 80% to attain significance at a .05 level. Since no other data was available for either agglutination of *Aa* or killing of Gram-positive bacteria with saliva from LAP subjects we used the calculations derived from these Lf experiments to choose a subject sample size of 10 for each group.⁹

Ten *Aa*-positive subjects healthy at screening who developed bone breakdown (LAP/AA), 10 *Aa*-negative subjects who remained healthy (H), and 10 *Aa*-positive subjects who remained healthy (H/AA) were enrolled. Three other LAP/AA subjects who had bone loss on entry had limited saliva, which was used in the Lf assays only. Comparison of the three groups was made using an ANOVA followed by a Tukey-Kramer pair-wise post-hoc statistical analysis. Significance was set at $p = 0.05$ level.

3. Results

3.1 Demographics and salivary collections

The demographic information shown in Table 1 reports on the data obtained at the screening visit. In the case of Lf experiments we had three additional subjects who had disease at screening (mean age 16.5) and these three were added to the analysis. Overall there were 18 females and 12 males in the study. The mean overall age was 14.96 ± 3.3 . Saliva was collected after lunch period. While we did not evaluate salivary flow rate in a consistent

manner we allowed 3 minutes for saliva collection and recorded the amount collected. Of the 30 subjects whose saliva was used in this study the amounts collected varied from 4 ml to 5 mls. Between group differences were not significant.

3.2 Salivary lactoferrin levels and iron content

Lf levels were low in subjects who had *Aa* and remained healthy ($p < 0.05$) while levels were lower in subjects who had *Aa* and developed disease prior to disease ($p < 0.05$; Fig 1A). Lf protein levels were elevated after bone loss. These findings agree with the published literature.^{9,18} Lf iron levels were 3.6 ± 1.7 ng Fe/ μ g of Lf in subjects who were *Aa*-positive and developed LAP prior to detection of bone loss and these levels remained low (4.1 ± 3.1 ng Fe/ μ g of Lf) after bone loss was detected ($p < 0.05$; Fig 1B). In comparison Lf iron levels were considerably higher in both H/AA and H subjects ($p = 0.01$).

3.3 Salivary agglutinating activity

Salivary agglutinating activity was assessed in three ways (Fig 2 A, B and C). In the first assessment agglutinating units were expressed for a smooth non-aggregating strain of *Aa* (the JP2 strain) as measured first by CFU's/ml and then expressed as a percentage of agglutination as compared to the starting dose of *Aa* cells added (Fig 2 A). Starting cells mixed with saliva from healthy *Aa*-negative subjects (H) agglutinated at a level of $0.06 \pm 0.03\%$. Comparison between cells agglutinating in PBS as compared to cells run in conjunction with healthy *Aa*-negative (H) saliva showed no difference in the percent of cells that agglutinated ($p=0.1742$). In contrast, *Aa* cells mixed with saliva from healthy *Aa*-positive subjects (H/AA) resulted in agglutination of $57.7 \pm 16.2\%$ which when compared to healthy *Aa*-negative subjects (H) or the PBS control showed a significant difference ($p < 0.001$). *Aa* cells mixed with saliva from *Aa*-positive diseased subjects (LAP/AA) resulted in agglutination of $12.6 \pm 3.8\%$. When saliva from LAP subjects (LAP/AA) was compared to saliva from *Aa*-negative healthy subjects (H) or PBS treated cells no significant difference was seen.

The pattern seen above remained similar when this data was normalized for percent agglutination per 100 μ g of sIgA (Fig 2B). Thus saliva from healthy subjects who were *Aa*-negative (H) showed 0.02% agglutination while saliva from LAP subjects (LAP/AA) showed 6.7% agglutination. These results were not significantly different. Agglutination occurred at a level of $23.9 \pm 2.6\%$ when saliva from healthy *Aa*-positive (H/AA) subjects was compared to saliva from either diseased *Aa*-positive (LAP/AA) subjects or *Aa*-negative healthy (H) subjects and normalized to percent agglutination per 100 μ g of sIgA. The level achieved was significantly greater than that seen in saliva from either of the two other groups (Fig 2 B; H/AA vs LAP/AA, $p = .009$; H/AA vs. H, $p = 0.0018$).

Saliva from a subset of 9 subjects (3 subjects from each group) was used to determine the contribution of sIgA to this agglutination process. Pre-treatment of saliva from *Aa* subjects that remained healthy (H/AA) or saliva from *Aa* subjects who developed LAP (LAP/AA) with Protein A to remove any contribution by IgG had no effect on the level of agglutination. In contrast, pre-treatment of saliva with Protein L, designed to remove IgA from the saliva, dramatically decreased the aggregation of the JP2 strain (Fig 2C). Data from

healthy (H) subjects is not shown because no agglutination took place initially in untreated H saliva and no changes were seen when either Protein L or A were added to pre-treat the H saliva.

3.3 Salivary anti-bacterial activity

We had previously shown that saliva from healthy *Aa*-negative subjects killed *Aa* but had no effect on *S. mutans* viability.¹⁴ These results were confirmed in the current study and thus 98.6% of *S. mutans* in CFUs/ml survived (vs. PBS control) in the saliva from the *Aa*-negative healthy (H) group (Fig. 3). Results also showed that only $25.1 \pm 7.6\%$ of *S. mutans* survived in saliva from *Aa*-positive LAP subjects. Thus LAP/AA saliva was $83.4 \pm 12.8\%$ more active in killing *S. mutans* than saliva from healthy subjects who did not harbor *Aa* (H); $p < 0.0001$. Further, healthy subjects with *Aa* (H/AA) had $51.2 \pm 9.3\%$ more killing activity when compared to saliva from healthy subjects devoid of *Aa* (H/AA vs. H; $p < 0.0022$). Saliva derived from diseased subjects with *Aa* (LAP/AA) showed $32.2 \pm 13.3\%$ more killing activity against *S. mutans* than saliva obtained from subjects with *Aa* who remained healthy (H/AA). This comparison between *Aa* healthy subjects (H/AA) and *Aa* diseased subjects (LAP/AA) showed a trend that approached significance $p = 0.06$ (Fig. 3A). Salivary effects on *S. sanguis* and killing of *Lactobacillus* were also evaluated Fig. 3B. In this case saliva from healthy *Aa*-negative (H) subjects killed $2.2 \pm 3.6\%$ of the *S. sanguis* population and $2.3 \pm 9.4\%$ of the *Lactobacillus* population. Saliva from healthy subjects who were *Aa*-positive (H/AA) killed $52.0 \pm 9.2\%$ of the *S. sanguis* population and $58.6 \pm 10.1\%$ of the *Lactobacillus* population. Saliva from the *Aa*-positive LAP subjects (LAP/AA) killed $85.9 \pm 9.2\%$ of the *S. sanguis* population and $91.7 \pm 10.1\%$ of lactobacilli.

In summary, saliva from LAP subjects was significantly more active in killing Gram-positive microbes than saliva from *Aa*-positive subjects who remained healthy (H/AA) for both *S. sanguis* and *Lactobacilli* ($p < 0.0001$). Saliva from healthy *Aa*-positive subjects who remained healthy (H/AA) was significantly more active against these bacteria than healthy *Aa*-negative subjects (H/AA vs. H; $p < 0.002$). Further saliva from LAP *Aa*-positive subjects (LAP/AA) was more active than saliva from *Aa*-positives who remained healthy (H/AA). This activity achieved significance for *S. sanguis* and approached significance for *Lactobacilli* and *S. mutans* ($p=0.06$).

4. Discussion

Our goal in this study was to determine whether there was a salivary activity profile that could distinguish between individuals who had *Aa* and remained healthy as compared to those subjects who had *Aa* and developed bone loss (LAP). Our assessment divided the salivary activity profile into three components; salivary lactoferrin iron levels, salivary agglutinating activity, and salivary killing activity.

Lf is well known as a multifunctional salivary glycoprotein that has anti-inflammatory, anti-bacterial, anti-viral activity.¹⁹ Lf is best known for its ability to sequester iron (hence the suffix ferrin) so as to prevent utilization of this important nutrient required by many bacteria for survival.¹⁹ With respect to *Aa* Lf has been shown to interfere with *Aa* binding to mucosal surfaces.^{7,8} It was also shown that Lf with reduced iron had no effect on *Aa* binding to

BECs; however, when Lf was saturated with iron it could block *Aa*'s ability to bind to BEC surfaces *in vitro* by up to 85%.⁸ In a companion report saliva from LAP subjects was found to have high levels of Lf protein but low levels of Lf iron after LAP had occurred suggesting that this Lf would not block *Aa*'s binding to mucosal tissue.⁹

In the current study we had access to saliva prior to detection of disease and after disease had occurred. In agreement with studies of periodontal disease and other mucosal infections our studies show that Lf levels are elevated post-infection suggesting that innate mechanisms are responding to dampen the microbial challenge. Our current data in agreement with previous data also shows that post-infection Lf iron levels remain low.⁹ Exactly why Lf iron levels do not become elevated post-infection was not the goal of this study but deserves further exploration.

In the context of the current study we are the first to show that both total Lf and Lf iron levels are significantly reduced prior to disease in subjects who have *Aa* at screening and then develop LAP as compared to subjects who have *Aa* and remain healthy. These findings suggest that saliva with low levels of Lf and low levels of iron would have little to no effect in blocking binding of *Aa* to soft tissue thus permitting *Aa* to attach. In contrast, *Aa* carriers who remain healthy have Lf iron levels that are relatively high suggesting that this Lf would block *Aa* colonization of soft tissues in the H/AA group. The effect of reduced soft tissue colonization by *Aa* on repopulation of hard tissue in the H/AA group is worth further examination.

A second salivary activity that we studied was agglutination. Salivary activity that causes agglutination can influence plaque formation.^{12,20,21} Salivary agglutinating activity can cause *Aa* to form large aggregates.¹² This agglutination process could facilitate removal and swallowing of *Aa* thus reducing its ability to be retained in the oral cavity. The data in this report suggests that IgA is the agglutinating factor in healthy *Aa*-positive subjects (H/AA). It further suggests that IgA fails to cause *Aa* to clump or agglutinate efficiently in LAP in contrast to *Aa*-positive subjects who remain healthy (H/AA).

Tooth attachment in *Aa* is known to be mediated by the *tad* and *flp* genes that allow *Aa* to form massive tooth related biofilms.²² It has been shown in-vitro that pre-treatment of tooth surfaces with saliva from healthy subjects reduces the binding of *Aa*-induced biofilm formation.²³ The results presented herein support this in vitro data and indicate that saliva from H/AA subjects can limit or reduce *Aa* biofilm development.²³ Our results indicate that healthy subjects who are *Aa*-positive and who remain healthy have saliva with a high level of agglutinating activity. Specific ways in which IgA modulates *Aa* agglutination needs to be explored in greater depth. Future studies should take into account salivary flow rates and how they may influence the agglutinating pattern.

A third salivary activity that we studied was the direct killing of bacteria. Salivary killing of plaque bacteria could play an important role in *Aa* survival in developing plaque by reducing bacteria that compete with *Aa* for nutrients.¹⁴ Bacterial colonization of tooth surfaces occurs in a temporally and spatially organized manner.^{24,25} Bacteria that attach to tooth surfaces do so as a result of bacterial affinities that also depend on electrostatic and hydrophobic

interactions.²⁶ Moreover, nutritional factors play an important role in successive events that lead to a compact biofilm community of microorganisms.^{27,28} It is clear that when plaque develops on a clean tooth surface there are specific bacteria that dominate. It is also clear that *Aa* is more fastidious and nutritionally dependent than some of the more competitive Gram-positive pioneer colonizers.^{29,30} Therefore *Aa* survival in the oral cavity would benefit from salivary activity that interferes with growth of the more hardy pioneer Gram-positive bacteria. This form of salivary interference should enhance the expansion of *Aa* colonies on the tooth surface.¹⁷ Our data indicates that subjects who develop *Aa*-induced disease have saliva that kills competing Gram-positive plaque bacteria at a level significantly greater than *Aa*-negative controls who remain healthy.¹⁴ Moreover, that level is greater than that seen for *Aa*-positives that remain healthy although this difference did not achieve significance at the 0.05 level for all bacteria studied (the level was 0.06 for *S. mutans* and *Lactobacillus*). More work is needed to better define the factor(s) in saliva that contribute to the reduction of competing Gram-positive bacteria found in the early plaque biofilm.

Overall these results suggest that saliva from LAP patients differs from saliva from *Aa*-carriers who remain healthy. Recently we have isolated a salivary substance that kills *Aa* in vitro from a periodontally healthy adolescent devoid of *Aa*.³¹ This substance, cystatin SA, is found in high concentrations in saliva from healthy subjects and in sublethal concentrations in subjects who have LAP.^{31,32} Of the three salivary activity parameters examined in this study, Lf iron levels, IgA agglutination, and salivary killing of Gram-positive bacteria, the Lf iron levels per unit of total Lf appear to show the clearest distinction between *Aa*-carriers who remain healthy and those who develop disease. Low levels of Lf iron would allow for greater *Aa* colonization of soft tissue. However, we have also shown that LAP subjects have saliva that causes less agglutination of *Aa* and kills more of the competing Gram-positive plaque biofilm bacteria. Based on the three salivary activity parameters identified in this study one could predict that *Aa*-carriers who remain healthy would have less retention of *Aa* as compared to *Aa*-carriers who develop LAP.

We conclude that specific factors in saliva can be used to differentiate between subjects who are *Aa*-positive at screening and develop LAP (LAP/AA) from subjects who are *Aa*-positive at screening and who remain healthy (H/AA). In the future we will determine whether these salivary factors can be defined more precisely and used to predict the initiation of *Aa*-induced LAP in a prospective study design.

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Abbreviations

Aa Aggregatibacter actinomycetemcomitans

LAP	localized aggressive periodontitis
Lf	lactoferrin
sIgA	salivary immunoglobulin A
BECs	Buccal epithelial cells
PBS	phosphate buffered saline
CFU	colony forming units

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A

B

Fig. 1. Salivary lactoferrin (Lf) levels and iron concentrations in saliva from different subject populations

A. Level of Lf in $\mu\text{g}/\text{dl}$ found in saliva in subjects with varying periodontal health. Lf in *Aa*-negative healthy subjects (H) was $2121 \pm 187 \mu\text{g}/\text{dl}$, *Aa*-positive healthy subjects (H/AA) was $862 \pm 776 \mu\text{g}/\text{dl}$; *Aa*-positive LAP subjects (LAP/AA) prior to disease was $268 \pm 81 \mu\text{g}/\text{dl}$. Lf levels in *Aa*-positive subjects after disease (LAP/AA on furthest bar to right) was higher than that seen prior to disease. Bars with differing letters were significantly different from each other. Thus *Aa*-negative healthy (H) and LAP subjects after disease were not different but Lf levels in *Aa*-positive healthy subjects (H/AA) was significantly higher than LAP subjects prior to disease (LAP/AA:C) and these were both lower than *Aa*-negative (H) and *Aa*-positive subjects (H/AA) who remained healthy ($p < 0.05$ in all cases).

B. Level of iron concentration in salivary lactoferrin in ng of Fe per μg of Lf. Iron levels were lowest in LAP subjects (LAP/AA) both before and after disease was detected. Subjects who were *Aa*-negative and healthy (H) had iron concentrations at $65 \text{ ng Fe}/\mu\text{g Lf}$ while *Aa*-positive healthy subjects (H/AA) had $150 \text{ ng Fe}/\mu\text{g Lf}$. Subjects with disease (LAP) before breakdown and after breakdown (LAP/AA) had 3.6 ± 1.7 and $4.1 \pm 3.1 \text{ ng Fe}/\mu\text{g Lf}$

respectively and these results were significantly lower than that seen in healthy subjects ($p < 0.001$).

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A

b

C

Fig. 2. Effect of saliva on agglutination of

A. Colony Forming Units of *Aa* per ml that agglutinates after treatment with saliva from different subject populations. Data presented as percent of cells agglutinated of total starting cell dose. Controls grown in PBS agglutinated at all level 0.01%. Saliva from healthy *Aa*-negative subjects (H) caused *Aa* agglutination at a level of 0.06 ± 0.04 %. Saliva from healthy *Aa*-positive subjects (H/AA) caused *Aa* agglutination at a level of 57.7 ± 16.2 %. Saliva from LAP subjects (LAP/AA) caused *Aa* agglutination at a level of 12.6 ± 3.8 %. Comparison of H vs C; $p = 0.174$; H/AA vs. C; $p = 0.008$; LAP/AA vs. C; $p = 0.68$; H vs H/AA; $p < 0.001$; H vs LAP; $p = 0.447$; H/AA vs LAP/AA; $p = 0.005$.

B. *A. actinomycetemcomitans* agglutination caused by saliva from different subject populations normalized to the concentration of sIgA.

All data presented as a percentage of agglutination per 100 $\mu\text{g/sIgA}$. When this data was normalized for % agglutination per 100 $\mu\text{g/sIgA}$ the pattern of agglutination remained similar to that seen in Fig 2A. Thus healthy *Aa*-negative subjects (0.03%) and *Aa*-positive LAP subjects (LAP/AA; 6.7%) were not significantly different. However, *Aa*-positive subjects who remained healthy (H/AA; 23.9 ± 2 %) had significantly more agglutination as compared to other groups (HAA vs Healthy; $p = 0.018$; H/AA vs LAP; $p = 0.009$).

C. A. actinomycetemcomitans agglutination in saliva pre-treated to remove sIgA. Data presented as a percentage of starting dose of *Aa* cells added to saliva. When saliva from H/AA subjects or LAP/AA subjects was pre-treated with Protein A, no reduction in agglutination was seen. When saliva from H/AA or LAP/AA subjects was pre-treated with Protein L, agglutination was reduced significantly. In this case H/AA saliva induced agglutination of *Aa* was 66.7%, while LAP/Aa saliva agglutinated at a level of 10.5%.

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A

b

Fig. 3. Effect of saliva on selected Gram-positive bacteria

Presented as a percentage of bacteria killed by saliva derived from healthy *Aa*-negative subjects (H), healthy *Aa*-positive subjects (H/AA) and *Aa*-positive subjects with localized aggressive periodontitis (LAP/AA).

A. *S. mutans*: Saliva from H subjects reduced *S. mutans* growth in CFUs/ml by $1.45 \pm 3.8\%$; saliva from H/AA subjects reduced *S. mutans* growth in CFUs/ml by $52.7 \pm 39.4\%$, and saliva from LAP/AA subjects reduced growth by $84.9 \pm 20.1\%$. LAP/AA vs H; $p = 0.0001$; H/AA vs H; $p = 0.002$; LAP/AA vs H/AA; $p = 0.06$.

B. *S. mutans*, *S. sanguis* G9B, *Lactobacillus*: *S. mutans* data is shown in the first bar on the left, *S. sanguis* data is shown in the middle bar, data for *Lactobacillus* is shown in the last bar on the right. *S. mutans* data presented in Fig 3A is re-presented here for comparison to other Gram-positive bacteria. Saliva from H subjects reduced *S. sanguis* growth by $2.2 \pm 8.6\%$; saliva from H/AA reduced *S. sanguis* growth by $52 \pm 9.2\%$; saliva from LAP/AA subjects reduced growth by $85.5 \pm 9.2\%$. LAP/AA vs H; $p = 0.001$, H/AA vs H; $p = 0.0025$, LAP/AA vs H/AA; $p = 0.045$. Saliva from H subjects reduced *Lactobacillus* growth by $2.3 \pm$

1.8%; saliva from H/AA subjects reduced *Lactobacillus* growth by 56.9 ± 42.2 %, and saliva from LAP/AA subjects reduced growth by 91.8 ± 22.1 %. LAP/AA vs H; $p = 0.001$, H/AA vs H; $p = 0.0024$, LAP/AA vs H/AA; $p = 0.06$.

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Table 1

Demographics

Ethnicity	Gender	Number	Age
African American	Male	10	14.33±1.58
	Female	10	14.50±0.93
Hispanic	Male	2	16.00±0.00
	Female	5	15.00±2.00
Not Reported	Male	0	-----
	Female	3	14.50±2.12
TOTAL		30	14.96±3.26