ORIGINAL REPORT: EPIDEMIOLOGIC RESEARCH

Arginine Metabolism in Supragingival Oral Biofilms as a Potential Predictor of Caries Risk

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Abstract: Introduction: Ammonia production via the arginine deiminase system (ADS) of oral bacteria can function to reduce the cariogenicity of oral biofilms by neutralizing glycolytic acids that cause tooth demineralization.

Objectives: This cohort study investigated the relationship between ADS activity and bacterial profile changes of supragingival biofilms with caries experience among children over time.

Methods: A total of 79 children aged 2 to 7 y at baseline were assessed every 6 mo for a period of 18 mo. Children were grouped as caries free (CF), caries active with enamel lesions (CAE), or caries active with dentin lesions (CA). Supragingival plaque samples were collected from caries-free surfaces (PF) and from enamel (PE) and dentin (PD) lesions. Plaque ADS activity was measured by monitoring citrulline production from arginine and compared with ribosomal 16S rRNA-

derived taxonomic profiles for the same samples.

Results: At baseline, 37% of the children were CF, 34% CAE, and 29% CA. At 18 mo, 26% were CF, 41% CAE, 23% CA, and 10% were caries experienced (new restorations but no caries activity). Throughout the study period, ADS activity was significantly higher in the CF group than the CA group ($P < 0.0001$), and ADS activity in the PF samples was significantly higher than in the PE and PD samples ($P < 0.0001$). Distance-based redundancy analysis showed that the bacterial communities could be differentiated when plaque samples are grouped into levels of high and low ADS activity.

Conclusions: There is a positive correlation between caries activity and low arginolytic capacity of the supragingival oral biofilms of children and tooth surfaces over time. Measurements of arginine metabolism via ADS may be useful to differentiate the caries risk of individuals and tooth surfaces.

Knowledge Transfer Statement:

Findings from this study support the development of new strategies for caries risk assessment and prevention based on modulation of the virulence of the oral microbiome through arginine metabolism in supragingival biofilms.

Keywords: dental plaque, dental caries, dental caries susceptibility, bacteria, risk factor, child health

Introduction

The development of caries lesions involves a dynamic biological process in which acids produced by bacterial glycolysis of dietary carbohydrates in oral biofilms, also called *dental plaque*, cause demineralization of dental hard tissues (Marsh 2006). Since low pH environment is a primary determinant, metabolic activities that help to promote a neutral pH in oral biofilms may have a strong inhibitory effect of the caries process. Early *in vitro* studies identified the amino acid L-arginine as

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A supplemental appendix to this article is available online.

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the main component responsible for the pH-raising effect of saliva (Kleinberg et al. 1979; Kanapka and Kleinberg 1983; Wijeyeweera and Kleinberg 1989). Arginine enters the mouth through dietary components but is also naturally produced by the human body via protein turnover and de novo arginine synthesis from citrulline. In supragingival biofilms, arginine is metabolized mainly by the arginine deiminase system (ADS) of certain oral bacteria to produce citrulline, ornithine, CO₂, ATP, and ammonia (Burne and Marquis 2000).

Robust evidence from *in vitro* (Stephan 1944; Turtola and Luoma 1972; Kleinberg 1978, 2002; Margolis et al. 1988; Sissons and Cutress 1988; Imfeld et al. 1995) and clinical (Nascimento et al. 2009; Nascimento et al. 2013) observations provides support that ammonia production via the oral bacteria functions to reduce the cariogenicity of oral biofilms. Specifically, ammonia production via ADS inhibits tooth demineralization by neutralizing glycolytic acids and favoring the growth of a desirable microflora that is compatible with dental health. Arginine has also been shown to affect the assembly of biofilm matrix (He et al. 2016), bacterial coaggregation (Ellen et al. 1992; Kamaguchi et al. 2001), cell-cell signaling (He et al. 2016), and adhesion of the caries pathogen *Streptococcus mutans* to tooth surfaces (Sharma et al. 2014). Our clinical studies revealed a positive correlation between caries activity and low arginolytic capacity of the supragingival microbial populations of adults and children (Nascimento et al. 2009; Nascimento et al. 2013). Novel therapies seeking to provide arginine as a substrate for ammonia production in oral biofilms may have great cost-effective potential for at-risk populations. In fact, toothpaste or mints containing arginine were demonstrated to be highly effective at inhibiting caries initiation and progression (Acevedo et al. 2005; Acevedo et al. 2008; Kraivaphan et al. 2013; Li et al. 2015).

In our recent study, we used Human Oral Microbe Identification Using Next

Generation Sequencing (HOMINGS) to explore the association between caries activity and the bacterial profiles of site-specific supragingival plaque samples, which revealed that the microbiome of healthy tooth surfaces differs substantially from that found during caries activity (Richards et al. 2017). Taxa most commonly found in the plaque of carious dentin included *S. mutans* and other acidogenic/aciduric species, such as *Scardovia wiggsiae*, *Parascardovia denticolens*, *Veillonella parvula*, and *Lactobacillus salivarius*. Taxa most commonly found in the plaque of healthy tooth surfaces included *Streptococcus sanguinis*, *Lautropia mirabilis*, *Abiotrophia defectiva*, *Corynebacterium durum*, and *Rotbia aeria*. This microbiome study strengthens the well-known associations of *S. mutans* with caries and *S. sanguinis* with health. Notably, a key property of *S. sanguinis* and other bacteria present in higher proportions in health-associated supragingival biofilms is their ability to produce ammonia via ADS, which has a positive impact on biofilm pH homeostasis (van Houte et al. 1994; Lingstrom et al. 2000; Becker et al. 2002; Aas et al. 2008; Gross et al. 2010; Crielaard et al. 2011). Altogether, significant findings over the past decade support the continuing investigation of the microbiological and functional bases for differences in the rates of arginine metabolism by oral biofilm communities in health and disease. Accordingly, the objective of our present study was to examine the relationship between ADS activity and bacterial profile changes of supragingival biofilms with caries experience among children over time. The study hypothesis was that ADS activity and bacterial profile change over time as a function of children caries status.

Methods

Study Design

This cohort study was designed in accordance with the STROBE guidelines. To determine the sample size, power

analyses (PROC POWER in SAS 9.3; SAS Institute) were performed with data from our previous studies (Nascimento et al. 2009; Nascimento et al. 2013). The analyses showed that a minimum of 60 children were required to address the aims of this study with a power of 80% ($\alpha = 0.05$). Considering the potential attrition rate, a total of 79 children aged 2 to 7 y were recruited for this study and assessed prospectively for 18 mo (baseline and 6, 12, and 18 mo with 5- to 7-d variation). The study population was a random selection representative of the target population. Children were recruited via study advertisements and from the pediatric dental clinics of the College of Dentistry, University of Florida, from January to August 2013. Baseline assessments started in April 2013, and 18-mo assessments were completed in February 2015.

Participants and Eligibility Criteria

Informed consent was obtained from parents or legal guardians of each child under a protocol approved by the Institutional Review Board of the University of Florida Health Science Center. The selection process excluded children who: 1) were treated with antibiotics within the past 3 mo, 2) were taking any medication on the days of the study visits, or 3) had orthodontic appliances. Parent-administered questionnaires were used to collect information on covariates that may be associated with ADS activity and caries: 1) children's demographic background (age, sex, and race), 2) socioeconomic status (parents' income and housing condition), 3) children's oral health practice (feeding histories, diet habits, oral hygiene measures, and dental attendance), and 4) systemic diseases.

At baseline, children were grouped by caries status as follows: caries-free (CF), with no clinical evidence of caries experience (decayed, missing, and filled teeth [DMFT] = 0); caries active with active enamel caries lesions only (CAE; DT = 0, MFT ≥ 0); and caries active with at least 2 active and unrestored dentin

caries lesions (CA; DT ≥ 2 , MFT ≥ 0). The caries status was then reassigned at each study visit. During the study, parents received education on caries prevention, and children were enrolled in dental care programs for restorative treatment when needed. Thus, the caries-experienced (CE; DT = 0, MFT ≥ 1) group was created for the consecutive study visits to include children presenting new restorations but no active lesions.

Caries Diagnosis

At each study visit, caries lesions were detected and diagnosed by a calibrated examiner using the visual criteria of the International Caries Detection and Assessment System (ICDAS; Ekstrand et al. 2007). Lesion activity was determined by clinical appearance, plaque stagnation, and tactile sensation (Nascimento et al. 2013). Teeth were examined before and after removal of dental plaque, as well as before and after being dried with compressed air for 5 s. The range of ICDAS scores (0 to 6; Ekstrand et al. 2007) as a function of caries status group were CF and CE (no activity, ICDAS = 0), CAE (active lesions, ICDAS = 0 to 3), and CA (active lesions, ICDAS = 0 to 6). The threshold for the CA group was the presence of at least 2 ICDAS scores of 5 or 6 (cavitated dentin lesions).

Sample Collection

Children were required to refrain from oral hygiene procedures for at least 8 h prior to collection of supragingival plaque samples. At each study visit, plaque was collected separately from tooth surfaces that were caries-lesion-free (PF; ICDAS = 0) or had active caries lesions in enamel (PE; ICDAS = 1 to 3) or dentin (PD; ICDAS ≥ 4), with the use of sterile periodontal curettes as previously described (Nascimento et al. 2013). PF was collected from all children, whereas PE was collected from the CAE and CA groups and PD from the CA group. To profile the bacterial communities, the samples were grouped as CF-PF, CAE-PF, CAE-PE, CA-PF, CA-PE, and CA-PD.

Plaque ADS Activity

Plaque ADS activity was measured by monitoring citrulline production from arginine with a validated protocol (Liu et al. 2008). Briefly, plaque samples were dispersed by external sonication for 2 cycles of 15 s, with cooling on ice during the interval. Plaque samples were then washed once with 10mM Tris-maleate buffer (pH 6.0), harvested by centrifugation, and resuspended in 115 μ L of the same buffer. Samples were then permeabilized by vortexing them with 6 μ L of toluene:acetone (1:9) for 2 cycles of 1 min, with cooling on ice during the interval. Permeabilized plaque was used to measure ADS activity in a reaction mixture containing 1M arginine, 0.1M hexanoic acid, and 0.5M Tris-maleate buffer (pH 6.0) for 90 min at 37°C. ADS activity was normalized to protein content and defined as nanomoles of generated citrulline: $[\text{min} \times (\text{mg protein})]^{-1}$.

Bacterial Community Profiles

ADS activity for each sample was paired with its respective 16S rRNA derived taxonomic profile previously generated by Richards et al. (2017). Briefly, HOMINGS (<http://homings.forsyth.org>) was used to survey the bacterial profiles of 186 site-specific plaque samples that were collected from 55 of the participating children at baseline and 12 mo. Plaque DNA was purified with a protocol that includes overnight incubation in the presence of Ready-Lyse Lysozyme Solution (Epicentre) and the MasterPure DNA Purification Kit (Epicentre). Purified DNA was sequenced with the Illumina MiSeq Platform (Illumina). Sequencing of the V3 to V4 regions of 16S rRNA with primers described elsewhere (Belstrom et al. 2016) was performed at the HOMINGS Core Facility at the Forsyth Institute. The HOMINGS approach assigns taxonomy with a customized BLAST program called ProbeSeq, which contains sequences of species- and genus-specific 16S rRNA probes based on the Human Oral Microbiome

Database. Each probe represents a distinct operational taxonomic unit (OTU). Bacterial identification is based on the use of 638 oligonucleotide probes (17 to 40 bases) targeting individual oral bacterial species and/or a few closely related species and 129 genus-specific probes that identify closely related species within the same genus. The Appendix Table shows the description of the HOMINGS taxa, species, and genus probes.

Statistical Analyses

All data management and statistical analyses were performed with SAS 9.3. For descriptive analysis, distributions of percentages and means were calculated when appropriate. Student's *t* test or analysis of variance were used to test the differences of continuous variables, and chi-square test was used for categorical variables. The generalized estimating equations method (Gaussian model) was used to examine the association between ADS activity and caries status. Python scripts within the software package QIIME version 1.9.0 were used to analyze HOMINGS OTU counts. Alpha diversity rarefaction plots with the chao1 richness factor were generated for all samples (Chao 1984). Read counts for each sample were normalized by random subsampling of each sample. The subsampling threshold ($n = 34,621$) was the least number of reads in the sample. Normalized counts were used to calculate beta (Bray-Curtis) diversity measures (Bray and Curtis 1957). Samples were grouped according to ADS activity as follows. The 30 samples with the highest levels of ADS activity were classified as high; the 30 samples with the lowest levels, low; and all remaining samples, intermediate. A distance-based redundancy analysis was performed, and significant differences in beta diversity among the ADS groups were determined with a permutational multivariate analysis of variance test. The frequency of OTUs among ADS groups was tested for significant difference with a Kruskal-Wallis test. *P* values were generated with 10,000 permutations and corrected for

Figure 1. Arginine deiminase system activity by caries status and study visit: (A) children and (B) plaque. Values are presented as mean $\pm$ SD. CF: caries-free; CAE: caries-active with enamel caries lesions; CA: caries-active with dentin caries lesions; CE: caries-experienced; PF: plaque from caries-free tooth surfaces; PE: plaque from active, enamel carious lesion; PD: plaque from active, dentin carious lesions. * $P < 0.001$.

multiple testing with the false discovery rate.

Results

Of the 79 children recruited at baseline, 62 completed the 18-mo study visit (attrition rate, 21.5%). Attrition occurred mainly due to change of address but also to scheduling issues. At baseline, the mean age of the participating children was 4.3 y. Of the participating children, 58% were male and 42% were female; 78% were Caucasian, 15% were African American, and 7% were from other races. At baseline, children had either primary (72%) or mixed (28%) dentition and were CF (37%), CAE (34%), or CA (29%). At the 18-mo visits, 61% of children had mixed dentition, and were CF (26%), CAE (50%), CA (21%), and CE (3%; Appendix Fig. 1).

ADS activity of plaque samples ranged from 0.01 to 33.2 units (mg protein)⁻¹. Generalized estimating equations analysis indicated that age, race, sex, and dentition had no effect on the levels of plaque ADS activity. Throughout the study, ADS activity of the CF group was significantly higher than that of the CA group ($P < 0.0001$; Fig. 1A), and likewise for PF versus PE ($P < 0.0001$) and PD ($P < 0.0001$; Fig. 1B).

Alpha diversity rarefaction curves (Appendix Fig. 2) started to plateau at approximately 1,000 reads, suggesting

Figure 2. Constrained analysis of principal coordinates (CAP) of plaque bacterial communities grouped by arginine deiminase system activity with distance-based redundancy analysis. Plaque samples were grouped as high, moderate, and low according to the level of arginine deiminase system activity.

that the normalization threshold was adequate and that the analysis achieved a good representation of community diversity. Distance-based redundancy analysis showed that the bacterial communities could be differentiated when samples were grouped as high, intermediate, or low according to their levels of ADS activity (Fig. 2). Furthermore, there was a statistically significant difference between the high and low ADS activity groups ($P =$

0.0012). However, taxonomic profiles also indicated great diversity for the bacterial profiles, among and within the plaque groups, as illustrated in Figures 3 and 4 and Appendix Figures 3 and 4, which show the distribution of the 30 most frequent taxa among the plaque groups. Figures 3 and 4 also show the distribution of the most frequent taxa among the samples with the highest and lowest levels of ADS activity, respectively. The figures indicate the distribution of

Figure 3. Distribution of the 30 most frequent taxa among the 30 samples showing the highest arginine deiminase system activity. CF: caries-free; CAE: caries-active with enamel caries lesions; CA: caries-active with dentin caries lesions; CE: caries-experienced; PF: plaque from caries-free tooth surfaces; PE: plaque from active, enamel carious lesion; PD: plaque from active, dentin carious lesions. X-axis represents the samples codes and their respective ADS activity levels and Y-axis represents the frequency distribution of normalized taxon counts.

Figure 4. Distribution of the 30 most frequent taxa among the 30 samples showing the lowest arginine deiminase system activity. CF: caries-free; CAE: caries-active with enamel caries lesions; CA: caries-active with dentin caries lesions; CE: caries-experienced; PF: plaque from caries-free tooth surfaces; PE: plaque from active, enamel carious lesion; PD: plaque from active, dentin carious lesions.

a health-associated taxon (*S. sanguinis*) and 2 caries-associated taxa (*S. mutans* and *Veillonella dispar*). Appendix Figures 3 and 4 show the distribution of the

most frequent taxa among the CF-PF and CA-PD samples, respectively.

Figure 5 shows the distribution of the 60 most frequent taxa among

the ADS groups. Of these 60 taxa, 3 showed a statistically significant difference in distribution among the high, intermediate, and low ADS

Figure 5. Distribution of the 60 most frequent taxa among the 3 arginine deiminase system (ADS) groups. *A taxon with a significant difference in distribution among the ADS groups. The left chart shows the sum of normalized taxon counts for each ADS group, expressed as proportions of the total. The right chart shows the sum of normalized taxon counts for each ADS group. Digit codes next to taxon names are HOMINGS identifiers (Human Oral Microbe Identification Using Next Generation Sequencing). CA, caries active with dentin caries lesions CAE, caries active with enamel caries lesions CE, caries experienced CF, caries free PD, plaque from active dentin carious lesions PE, plaque from active enamel carious lesions PF, plaque from caries-free tooth surfaces.

groups: *Porphyromonas* sp. oral taxon 279 ($P = 0.040$), *Bergeyella* sp. oral taxon 322 ($P = 0.049$), and *Fusobacterium periodonticum* ($P = 0.049$). Three additional taxa showed a statistically significant difference

in distribution but were outside the top 60: *Actinomyces* sp. oral taxon 178 ($P = 0.008$, rank = 64), *Lachnoanaerobaculum umeaense* ($P = 0.020$, rank = 66), and *Prevotella intermedia* ($P = 0.024$, rank = 265).

Discussion

This study defines, for the first time, the arginolytic potential of supragingival plaque populations of children over time in the context of dental caries status.

Plaque bacteria from children and tooth surfaces in the CF group had consistently higher ADS activity as compared with those from caries-active children and carious tooth surfaces during the 18 mo of the study. The most straightforward explanation for the differences in ADS activity between health-associated and carious plaques may be related to the composition of the microbiomes inhabiting these different sites. It is also possible that the microenvironments in biofilms of caries-active individuals and caries-active tooth sites may not favor high levels of ADS expression or may contain inhibitory factors that decrease ADS expression or enzyme activity. Notably, bacterial heterogeneity in arginolytic capacity was associated with intra- and interspecies variation in the regulation of the ADS and peak levels of ADS activity, likely associated with evolution of adaptive strategies for acid tolerance and nutrient limitation (Huang et al. 2015). Nevertheless, our findings support the hypothesis that arginine metabolism in supragingival biofilms may greatly affect the resistance or susceptibility of the host to dental caries (Nascimento et al. 2013). They also suggest that measurements of arginine metabolism by plaque bacteria may be useful to differentiate the caries risk of tooth surfaces of individuals over time.

Measurements of ADS activity in supragingival plaque samples offer the opportunity for the design of novel and much-needed risk assessment tools for caries (Nascimento 2018). For further validation of plaque ADS activity as a caries risk assessment criterion, future clinical trials should be designed with an integrated model that includes 1) arginine metabolism as a caries-protective factor and 2) other recognized pathologic risk factors as predictors of caries. Conceivably, novel chair-side tests with plaque ADS activity as a caries risk assessment tool could be developed in the future (Nascimento 2018). Potential arginine-based approaches for caries intervention, to be used separately or in combination, include: 1) arginine supplementation as

a prebiotic; 2) arginine incorporation in oral care products as a therapeutic agent to modify plaque composition and biochemical activities; and 3) probiotic formulations composed of naturally occurring isolates that show high ADS expression, low cariogenic potential, and antagonistic properties against cariogenic bacteria. Long-term randomized clinical trials should be performed to further evaluate the effect of arginine supplementation in oral health.

Genome sequencing and other molecular techniques have revealed new levels of complexity and heterogeneity in the microflora and the nature of individual bacterial species (Mager et al. 2003; Corby et al. 2005; Aas et al. 2008; Crielaard et al. 2011). However, the implications of microbial community interactions in health and caries pathogenesis are still not well understood, including the contribution of bacterial community members in promoting health via mechanisms such as ammonia production. Our previous study with the same group of samples revealed that supragingival plaque harbors a highly diverse bacterial community and that the microbiome of healthy tooth surfaces differs substantially from that found when there is evidence of caries activity (Richards et al. 2017). It was also observed that the levels of bacterial alpha diversity increased progressively from the health-associated communities (CF-PF) to the communities representing the most advanced stage of caries (CA-PD; Richards et al. 2017). Notably, studies have not been consistent in determining whether higher community diversity can be associated with caries (Johansson et al. 2016) or dental health (Li and Wang 2002; Gross et al. 2010), but the sampling and sequencing methods used among these studies were also distinct. The sequencing method used in the present study targets hypervariable regions of the ribosomal 16S rRNA gene with high-throughput amplicon sequencing and is a common approach to studying bacterial communities. It should be acknowledged that this approach lacks

species-level resolution for certain taxonomic groups. Nevertheless, it provides a valuable broad overview of the taxonomic structure of the communities.

Despite considerable progress in dissecting the function and regulation of the ADS in certain oral bacteria, there remain serious deficiencies in our understanding of the distribution of this catabolic among bacterial species and their potential etiologic roles in the ecology of oral health and oral diseases. In the present study, plaque community profiles were correlated with ADS activity levels. Plaque communities from samples with the highest levels of ADS activity showed a distinctive bacterial profile as compared with those having the lowest ADS activity. Six taxa showed a statistically significant difference in their distribution among the high, intermediate, and low groups of ADS activity. At this point, no clear association can be made between these taxa and arginine metabolism. Future functional studies will be necessary to gain a deeper understanding of the relationship among the metabolic and community roles of these taxa, plaque arginine metabolism, and caries.

In conclusion, there is a positive correlation between caries activity and low arginolytic capacity of the supragingival oral biofilms of children and tooth surfaces over time. These findings enhance the understanding of microbial profile changes with differences in arginolytic capacity and the progressive stages of early childhood caries. Measurements of arginine metabolism via ADS may be useful for early identification of children at greater risk of developing caries. Our results can be rapidly translated into new strategies for caries risk assessment and preventive therapies based on modulation of the virulence of the oral microbiome through arginine metabolism.

Author Contributions

M.M. Nascimento, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; A.J. Alvarez, X. Huang, S.

Hanway, S. Perry, A. Luce, V.P. Richards, contributed to data analysis, critically revised the manuscript; R.A. Burne, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This work was supported by the National Institute of Dental and Craniofacial Research (K23-DE023579 and R01 DE25832). As a potential conflict of interest, some of the research work by M.M.N. and R.A.B. cited in this article has been supported by Colgate-Palmolive Inc., which holds patents on arginine-containing oral health care products. The other authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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