A Potential Biofilm Metabolite Signature for Caries Activity - A Pilot Clinical Study

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Introduction

Despite our knowledge of the basic concepts of dental caries, the most common chronic childhood disease [1], the interactions within the dental biofilm microbiota, which may serve as an important caries risk indicator [2-5] are not well understood. Although dental caries is clearly a microbial regulated disease, it is not a traditional transmissible infectious disease as it does not meet Koch's key postulates, that an infectious organism presence or absence is related to presence or absence of the disease. Presently, data concerning caries risk indicators are primarily based on the number and proportion of Streptococcus mutans which seem to augment caries prediction only when used in very young children [6].

The understanding of the correlation between metabolite signals of dental biofilm and caries is in its infancy. Takahashi and his group [7] focused on the central carbon metabolism, the Embden-Meyerhof-Parnas (EMP) pathway, the pentose-phosphate pathway, and the tricarboxylic cycle in supragingival biofilm and specific oral bacteria, S. mutans, Streptococcus sanguinis, Actinomyces oris, and Actinomyces naeslundii. The investigators used capillary electrophoresis and mass spectrometry to elucidate metabolic regulation in dental biofilms through the comparison of metabolite profiles between supragingival dental biofilm and representative biofilm bacteria preceding and following a glucose rinse [7,8]. Their study indicated that the central carbon metabolism is basically functioning in the microbiota of in vivo supragingival biofilm, and these pathways are most likely similar to the integration of metabolic pathways determined in representative in vitro biofilm bacteria, Streptococcus and Actinomyces. Furthermore, they found that rinsing with glucose resulted in lactate production in supragingival plaque, demonstrating an increase of metabolites upstream and a decrease of metabolites downstream (except pyruvate) of the EMP pathway. Another study by this group demonstrated that metabolite analyses can be used to detect metabolic regulation in in vivo supragingival plaque [8].

Understanding the physiological traits of bacteria in oral biofilms is key for the identification of new preventative or therapeutic measures to control dental caries [9]. Findings indicate that around 200 bacterial species/phylotypes can be found in the oral cavity by 16S DNA analysis [5,10] and that there may be pathogenic communities in the dental biofilm microbiome that significantly correlate with dental caries status [10,11]. Recently, a core functional microbiota of almost 60 species was identified [12]. The metabolic changes that occur in the diverse oral biofilm during the transition from a non-pathogenic to a pathogenic state may also prove to be important disease indicators.

Modern management of dental caries involves determining the caries risk status of the patient; detecting and assessing caries lesions at an earlier stage; making a diagnosis as to whether the disease is actually present; establishing a prognosis; applying intervention strategies focused on preventing, arresting and possibly reversing the caries process; and delaying restorative treatment to only when absolutely necessary [13]. Identification of at-risk active sites is a core component of the principles for the modern management of dental caries [13] and this is the biggest challenge faced by dentists. Currently there is no means to accurately determine if a site will develop a lesion, or if a lesion will progress to cavitation. The goal of this preliminary study was to identify potential site-specific metabolite profiles in caries-free and...
caries active areas (areas with a lesion progressing towards cavitation).

It was hypothesized that caries lesion activity is a reflection of the activity of the overlying dental biofilm; therefore, children that had caries-active lesions can be distinguished from children with no caries activity based upon the metabolite signal of their biofilm. A signature associated with dental caries status should enable a more accurate determination of lesion activity at the time of a dental examination. The results indicate that there is a potential distinct metabolite profile in the biofilm of caries active lesions.

Materials and Methods

A convenience sample of 15 children from public schools in Aguas Buenas, Puerto Rico who were enrolled in a parent study previously described [14-16] was recruited in June 2011 as approved by the IRB committees from Indiana University (IU-IRB #0068-15) and University of Puerto Rico (UPR-IRB#A1340107). Parental consent was obtained along with assent. For inclusion in the study, children had to have been enrolled in the parent study, be between 10-15 years of age, have no medical problem that contra-indicated participation, have been characterized as caries-active (at least one lesion progressed to cavitation in the prior year) or caries-free (no history of lesions or fillings) per the International Caries Detection and Assessment System (ICDAS) and allow collection of dental biofilm. The children were requested not to brush their teeth or eat after the last meal (dinner) the evening before dental biofilm collection. All children were seen in the morning and dental biofilm samples were collected after overnight fasting (day 1) and after a 1 minute rinse with 15 ml of 20% glucose rinse (day 2). Dental biofilm samples were collected from 15 children, 11 characterized as caries-active (ICDAS 1-3) and 4 characterized as caries-free (ICDAS 0). On average there were 4 active sites (C-A) per caries-active child, with two children having only one C-A site and one child having 10 C-A sites, and there were 10 samples collected from caries-free sites (C-F) for each child (caries-active or caries-free child). At each collection day, for each child, biofilm samples were combined in two different collection vials according to the surface's caries status (C-A or C-F), re-suspended in ice-cold 100% ethanol and analyzed regarding their metabolite profile by GC/MS after sorptive extraction at the Indiana University Institute for Pheromone Research.

GC/MS Method

The dental biofilm was weighed and re-suspended in 800 μl of 100% ethanol (Koptec, King of Prussia, PA) and stored at -20°C prior to the metabolite profiling using previously described methods [17, 18]. Each sample vial containing dental biofilm and ethanol was emptied to a 20 ml glass scintillation vial and the vial was filled to 20 ml with high-purity water (Omnisolv EM Science, Gibbstown, NJ). The sample was ultrasonicated for 15 minutes in the presence of 50 mg of ammonium sulfate (99.999%, Sigma-Aldrich, St. Louis, MO). Previous analyses have shown that ammonium sulfate amplifies the extraction recoveries of volatile compounds from biofilm samples, as has been previously seen with other difficult biological sample matrices. Subsequently, 8 ng of the internal standard (7-tridecanone from Sigma-Aldrich, St. Louis, MO) in 5 μl of methanol (J.T. Baker, Center Valley, PA) and a Twister™ stir bar (10 mm long, 0.5 mm film thickness, 24 μl polydimethylsiloxane volume, Gerstel GmbH (Mülheim an der Ruhr, Germany) was added in each vial for the 60 min sorptive extraction process (at 800 rpm) to enrich small organic metabolic products from the ethanol-water phase (20 ml) into the polymer phase (24 μl). After the extraction, the stir bars were placed in the Thermal Desorption Autosampler (TDSA) tubes for the GC/MS analysis. Samples were thermally desorbed in a TDSA automated system in a splitless mode, followed by injection into the GC column with a cooled injection assembly, CIS-4 (operating at the temperature range from -80 to 270°C). The separation capillary column in the analyses was DB-5MS (30 m × 0.25 mm, i.d., 0.25 μm film thickness) from Agilent Technologies Inc. (Wilmington, DE). The GC unit operated in the constant-flow mode. The GC/MS instrument used for the compound identification was the Agilent 6890 N gas chromatograph connected to the 5973i MSD mass spectrometer. Positive electron ionization (EI, 70 eV) mode was used with the scanning rate of 2.41 scans/s over the mass range of 41-350 amu. Total ion chromatograms (TICs) were measured for all the samples. Post-run selected ion chromatograms (SICs) at ion m/z 55 for the analytes of interest and the ion m/z 113 for the internal standard were recorded (Figure 1). The MSD transfer line temperature was set at 280°C. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively.

Statistical Analyses

The C-A and C-F sites were compared for metabolite profile considering differences in peak area ratios of the ion m/z 55 for the compounds 1-6 (Figure 2) in a cluster of branched alcohols and esters (26-45 min) and the ion m/z 113 for the internal standard (7-tridecanone, 50.01 min) in the corresponding run. After the peak area ratios were calculated and normalized further per unit weight (mg), multivariate Wilcoxon Signed Rank (WSR) tests [19] were applied for simultaneous assessment of the six compounds in four null hypotheses: (H1) there were no differences in peak area ratios for C-A sites and C-F sites among caries-active children without glucose rinse; (H2) there were no differences in peak area ratios for C-A sites and C-F sites among caries-active children with glucose rinse; (H3) there were no differences in peak area ratios for C-A sites in caries-active children with and without glucose rinse; and (H4) there were no differences in peak area ratios for C-F sites in all children with and without glucose rinse. A sensitivity analysis was conducted for the last hypothesis by applying the multivariate WSR test to peak area ratios from only caries-active children. The multivariate tests provide a degree of Type I error control. For each hypothesis, univariate WSR tests are conducted for the six individual compounds only if the corresponding multivariate test is statistically significant at the 0.05 level, which would indicate significant differences for one or more of the six compounds. The univariate tests use a Bonferroni-corrected significance level such that a p-value less than 0.05/6=0.0083 is considered statistically significant (S), a p-value>0.05 is not statistically significant (NS), and a p-value between 0.0083 and 0.05 is considered almost statistically significant (AS). Due to the small sample size, p-values for all WSR tests were based on the permutation distribution of the test statistic under the null hypothesis [20].

As a secondary analysis, multivariate linear models are fitted separately to the six compounds to simultaneously estimate the magnitudes of the effects of glucose rinse (yes/no) and caries presence (C-A vs. C-F) on the peak area ratios. In these models, a child contributes up to four peak area ratio values corresponding to glucose rinse (yes/no) and caries presence (yes/no). Due to skewness, the log10 transformation was applied to the peak area ratio after adding a 1 to address the presence of a zero outcome. All models were fit using SAS v. 9.3 Proc Mixed with Kenward-Roger degrees-of-freedom correction for small sample sizes [21] and a Kroncker-product covariance structure [22] to account for repeated measures (i.e., the quad-variate

Results

During the sample preparation for the analyses two C-A samples were lost. Demographics and clinical data of the subjects are presented in Table 1. Samples from C-A sites weighed on average 0.0012 g (range 0.0001 to 0.0025 g) and from C-F sites on average 0.0098 (range 0.034 to 0.0288 g). Reproducibility of the GC/MS method was 8.50% (relative standard deviation, RSD, n=10) calculated for the internal standard peak area of the ion m/z 113 at retention time of 50.01 min total ion chromatograms (TICs) for each sample were post-run simplified by initially filtering the data by several different selected ions (SICs) as shown in Figure 1 for the ion m/z 55. The described GC/MS data by filtering by the ion m/z 55 showed most clearly that dental biofilm collected from C-A sites with or without a previous glucose rinse exhibited a differential metabolic profile compared to samples from C-F sites (Figure 3). This was consistent across all subjects independent of the number of C-A sites present (1 or 10). The qualitative data analysis suggests that a special cluster of branched alcohols and esters is present at substantially higher intensity in biofilms of C-A sites versus C-F sites. This finding was demonstrated quantitatively when the peak areas of these compounds were normalized against an internal standard and per unit sample weight showing more pronounced metabolite amounts in samples from C-A sites, either in fasting conditions (V2, p=0.0205) or after glucose rinse (V3, p=0.0033) (Figure 4). Tentative identification of the compounds in the Figure 2 was based on the NIST library spectra (NIST Mass Spectral Search Program for the NIST/ EPA/ NIH Mass Spectral Library. Version 2.0 a., 2002). Possible compound structures include isooctanol (Rt 26.52 min), 2-ethyl-1-hexanol acetate (Rt 28.74), 3-methyl-1-heptanol or 6-methyl-1-heptanol (Rt 29.49 min), 2-propenoic acid octyl ester (Rt 32.38 min), 4,8-dimethylnonanol (Rt 39.40 min). However, the library matches were relatively low with 40-60 % probability.

In particular, the multivariate WSR test for H1 indicates that there
was an overall significant difference between C-A and C-F sites before glucose rinse (p=0.021). Similarly, the multivariate WSR test for H2 indicates that there was a significant difference between C-A and C-F sites after glucose rinse (p=0.019). For H3, the overall difference in peak area ratios with and without glucose rinse for C-A sites was not significant (p=0.412). For H4, there was a significant difference between peak area ratios taken before and after glucose rinse (p-value=0.028) for C-F sites among all children. However, in the sensitivity analysis for C-F sites from CA children only, the change from visit 2 (before glucose rinse) to visit 3 (after glucose rinse) was not significant (p-value=0.135).

According to the univariate WSR tests, C-A and C-F sites had significance differences before glucose rinse in alcohol 1 and alcohol 2 (with the same p=0.005), while adjusting for multiple testing the tests were almost significant for the two other alcohols and 2 esters. After glucose rinse, C-A and C-F sites had significance differences in alcohols 1, 2 and 3, and esters 1 and 2 (same p-value =0.005). The corresponding test was almost significant for Alcohol 4 (p-value=0.013). Finally, the univariate tests indicate that, the change from visit 2 to visit 3 for C-F sites (from all participating children) was significant only for Alcohol 1 (p-value=0.006) and the changes for the remaining three alcohols and ester 2 were almost significant. The change was not significant for ester 1.

For sound sites the comparison between the four alcohols and two esters indicated that there were no statistically significant differences between sound sites from caries-active children and caries-free children.

Table 2 shows that, on average, the differences in intensity of the normalized peak areas between C-A samples and C-F samples ranged from 0.40 to 0.70 on the log10 scale.

**Discussion**

It is known that the intake of dietary carbohydrates induces ecological shifts in dental biofilm microbiota. The low-pH environment created due to fermentation of these carbohydrates transits dental biofilm microbiota from a dynamic stability stage to an acidogenic/aciduric stage which might result in carious lesion initiation and progression [23]. These frequent events of acidification in dental biofilm induce an Acid Tolerance Response (ATR) in the microbiota raising the proportions of acid tolerant microorganisms [24]. In this
Figure 3: Inversely overlaid selected ion m/z 55 chromatograms at C-F (sound) and C-A (lesions) sites of two subjects after glucose solution rinse. Y-axis shows the abundance (intensity of the signal) and x-axis shows the elapsed time. A, B: subject 1; C, D: subject 2.
context, it is accepted that there is an increase in the proportion of non-mutans streptococci in dental plaque biofilm as analysed by five-

Figure 4: Averages of normalized peaks areas against internal standards and per mg of the sample weight in samples from C-A children from sound or lesion surfaces. Error bars indicate standard deviation (n=8).

Table 2: Estimates from multivariate models with transformation.

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<th>Effect</th>
<th>Estimate</th>
<th>SE</th>
<th>P-value</th>
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<td>Alcohol 1</td>
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<tr>
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<td>Intercept</td>
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<tr>
<td>Alcohol 3</td>
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<tr>
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Although the relationship between diet, frequency of exposure to sugars and dental caries has been clearly shown, it is important to consider that dental caries is a dynamic process arising as a result of a network of complex metabolic and synergistic and/or antagonistic interactions amongst the dental biofilm microbiota. Under these circumstances, bacterial acid adaptation likely plays an important role in the transition of biofilms from a healthy to a pathogenic state.

Conclusions

Gathering information on metabolite profiles will enable future research to address the functional significance of specific biochemical and regulatory pathways that are potentially associated with and contribute to the onset and development of dental caries. This pilot study indicates that there is likely a characteristic metabolomic signature for caries activity. Further studies are warranted to identify the metabolite signals in biofilm which can ultimately translate into a signature associated with at-risk sites with implications in caries risk assessment, caries diagnosis and caries management.

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