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Exploring The Effect Of Dentition, Dental Decay and Familiality On Oral Health Using Metabolomics

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Abstract

As a proof of principle, we used an untargeted global metabolic profiling of saliva to understand the biochemical processes associated with dental decay, dentition (primary and secondary tooth eruption) and familiality in a sample of 25 sibling pairs. Pairs were selected to represent four different combinations of dentition and tooth health: 1) both siblings with primary teeth and no decay (n=5); 2) both siblings with primary teeth and discordant for dental decay (n=6); 3) both siblings with primary teeth and dental decay (n=4); and 4) one sibling with primary teeth the other with mixed dentition and both with no dental decay (n=10). There was a strong effect of sibship on the metabolite profiles identified; this may reflect the effects of common genes, environment and behaviors, and shared oral microbial communities. Nested in the familial effects were associations of metabolite profile with dentition and with dental decay. Using three different analyses (Euclidean distance, hierarchical clustering and PCA using selected biochemicals) metabolite profiles of saliva from children with decayed teeth were more similar than the metabolite profiles of saliva from children with healthy (sound) teeth. Larger studies that include host behaviors, environmental factors, oral microbiota composition and structure, and host genetic predisposition are required to identify biomarkers for decay, and to estimate the relative contribution of host factors and oral microbes on risk of dental decay.

Keywords

metabolomics; dental caries; familiality

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1.0 Introduction

Dental caries is a dysbiosis of the oral microbial community that results from a change in the membership and structure of the resident microbiota (Marsh and Bradshaw, 1995). No single bacterial species is both a necessary and sufficient cause of dental caries, although sets of bacteria that are aciduric and acidogenic promote caries and are frequently detected in dental lesions (Takahashi and Nyvad, 2011). However, perturbations in microbiota alone are not sufficient to cause decay (Jarchum and Pamer, 2011): interactions between the complex microbial ecology of the mouth with host factors are important determinants of oral health and dental decay. Host factors include tooth shape and composition, salivary characteristics such as salivary flow and buffer capacity, and host behaviors (diet and oral hygiene). Host factors help shape the structure and membership of the bacterial communities that reside in the different niches in the oral cavity, including teeth, tongue and buccal surfaces.

Risk of dental caries is similar within family members, and specific human gene polymorphisms have been associated with dental caries risk (Shaffer et al., 2013; Suzuki, 1998; Zeng et al., 2013). Oral and plaque microbiota may also be similar: vertical transmission of microbiota from mother to infant establishes the initial microbiota in children (Collado et al., 2012), and transmission of caries-associated bacteria, *Streptococcus mutans*, from mother to child has been reported (Barnes et al., 2009). As children age, acquire teeth, begin eating solid food, and brushing their teeth, there are corresponding changes in the oral microbiota. Diet and dental hygiene behaviors affect microbiota composition and structure, are risk factors for dental caries, and are often similar within a family, making it difficult to separate the relative contributions of microbiota, genetic disposition and behavior on caries risk.

Bacterial metagenomic studies have deepened our understanding of the bacterial community composition and structure among those with and without dental decay. This characterization might, however, be somewhat misleading, as different members of the bacterial community can serve similar or even the same functions. One way to capture ongoing bacterial and host functions is to use metabolomics. Salivary metabolomics have demonstrated functional differences between health and periodontitis (Barnes et al., 2009), but we found no previous studies comparing global salivary metabolites patterns associated with health and dental caries.

As a first step in parsing the complex interaction between the human genome, oral microbiome, and environment, we conducted an exploratory study using an untargeted metabolomics screen of saliva from 50 children participating in the Center for Oral Health Research in Appalachia (COHRA) study, which is conducted jointly by the Universities of Pittsburgh, West Virginia, and Michigan. The purpose of this study was 1) to use an untargeted metabolomics screen to capture a snapshot of the host and bacterial processes ongoing in the oral cavity; 2) to determine if this metabolite snapshot can be used to identify differences in oral metabolism between children with healthy teeth and dental decay; and 3) to evaluate the effect of family in shaping oral metabolism.

2.0 Methods

2.1 Sample Collection and Metabolomic Profiling

We selected 25 sibling pairs that represent four different combinations of dentition and tooth health (Table 1). Samples were collected at least one hour after eating or drinking, using the Oragene Self-Collection Kit (DNA Genotek) or using a cheek swab and then preserved in an Oragene DNA kit (DNA Genotek). Participants were instructed not to eat or brush their

teeth for at least two hours before the screening (Polk et al., 2008). Collected samples were stored frozen at -80°C until thawed for testing.

An untargeted metabolomic profiling of all samples was conducted by Metabolon, Inc. following procedures described previously (Barnes et al., 2009; Evans et al., 2009; Ghannoum et al., 2011; Lawton et al., 2008). Each sample was accessioned into the Laboratory Information Management System (LIMS) at Metabolon and assigned a unique identifier and bar code. Quality control (QC) compounds were added to every sample to ensure data quality and to aid in the data curation procedure. In addition, we included 4 duplicated samples as technical replicates for quality control purposes. Metabolon was masked as to the identity of the replicates.

As described previous (Evans et al., 2009), samples were prepared for LC/MS and GC/MS platform by treating with methanol to precipitate protein with vigorous shaking for 2 minutes (Glen Mills Genogrinder 2000). After centrifugation the supernatant was removed and split into equal volumes. Aliquots of samples were vacuum dried and analyzed in Waters Acquity UPLC system and Thermo-Finnigan LTQ spectrometer including an electrospray ionization source and linear ion trap mass analyzer. Sample aliquots were reconstituted, one each in acidic or basic LC-compatible solvents containing 8 or more injection standards at fixed concentrations (to both ensure injection and chromatographic consistency). Extracts are loaded onto columns (Waters UPLC BEH C18-2.1 $\times$ 100 mm, 1.7 μm) and gradient-eluted with water and 95% methanol containing 0.1% formic acid (acidic extracts) or 6.5 mM ammonium bicarbonate (basic extracts). Samples for GC/MS analysis were dried under vacuum desiccation for a minimum of 18 hours prior to derivatization under nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). The GC column was 5% phenyl dimethyl silicone and the temperature ramp was from 60° to 340°C in a 17 minute period. All samples were then analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy daily. The instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites present in 100% of the samples.

The data analysis informatics components included data extraction into a relational database and peak-identification software; proprietary data processing tools for QC and compound identification; and a collection of interpretation and visualization tools for use by data analysts (Evans, et al., 2009). Peaks of the mass spec data files were identified using Metabolon's proprietary peak integration software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. A variety of curation procedures were used to ensure the quality of the data set available for statistical analysis and interpretation. Each compound was checked against Metabolon's in-house library of more than 1000 compounds for its identity. Those representing system artifacts, mis-assignments and background noise were removed. A data normalization step was performed to correct variation caused by instrument inter-day tuning differences and for the purposes of data visualization. Data points of each compound were normalized to have their median equal to 1.

The metabolite data used in the manuscript are available to the readers in the supplement.

2.2 Data Analysis

2.21 Comparison of Euclidean distance between participant pairs—We used the estimated Euclidean distance between participant pairs (i.e. any two participants regardless

of siblings or not) to assess whether the oral metabolites are more similar as a result of dental health status (decayed vs. healthy), dentition (primary vs. mixed) and familiarity (pairs of siblings vs. pairs of randomly picked participants). Euclidean distance is an effective method to measure the similarity of multivariate measures from samples, especially when the data are normalized to be on the same scales. We applied Kolmogorov-Smirnov test to compare the probability distributions of the Euclidean distances. We then used linear regression and ANOVA to assess whether metabolite profile difference is driven by the age difference between pair members, or by dentition for the sibling pairs discordant by dentition.

2.22 Hierarchical clustering within study groups—We further specifically evaluated the effects of familiarity, decay status and dentition within each group of sibling pairs using hierarchical clustering analysis based on the metabolite composition of each participant (package *pvclust* in software R, (Suzuki and Shimodaira, 2011)). The hierarchical clustering method uses the average-link method to agglomerate samples based on their metabolite profile, with the dissimilarity between samples calculated using the correlation method.

2.23 Effect of decay, dentition and family—To specifically assess the effect of dental decay and dentition on the metabolite profiles, we applied a permutation based multivariate analysis of variance (MANOVA) using the *ADONIS* function in package *vegan* in software R (Oksanen et al., 2013). This method allows us to partition variation from distance matrices constructed with the metabolite profiles. We assessed whether the variation in distance matrices due to dental decay status is significant among participants with and without dental decay; and similarly, we assessed whether the variation is significant among participants with primary and mixed dentition.

Finally, we tested the familial effect using Intra-Class Correlation (ICC) index, a commonly applied method in studies on heritability (Sedgwick, 2013). We calculated the ICC for each biochemical, using the sibling pairs within each group (Table 1) as replicates. The overall ICC statistics of all biochemicals provides us with a quantitative comparison of the familial effects among different groups given the effect of varied dental decay status and dentition of the sibling pairs.

2.24 Identify key biochemicals and pathways related to dental decay—We used one-way ANOVA to identify metabolites that differ significantly between participants with and without decay. We then applied Principle Component Analysis (PCA), a multivariate ordination method widely applied in microbiology (Ramette, 2007), to examine whether the metabolites collectively distinguish between participants with and without dental decay.

3.0 Results

We performed an untargeted salivary metabolomic screening to identify host and bacterial metabolites among 25 sibling pairs selected from families participating in a longitudinal study of dental decay in rural Appalachia. Pairs were selected to represent four different combinations of dentition and dental decay status (Table 1); all children with decayed teeth had primary dentition. Children with primary teeth averaged 3.0 years of age (when most children will have their complete set of primary teeth) and averaged 17.8 teeth. Those with mixed dentition were older (7.3 years) and averaged 19.9 teeth. The average number of decayed, missing and filled teeth for children with dental decay was 5.6.

In the untargeted metabolomics screen of saliva from the 25 sibling pairs, 282 biochemicals of known identity were detected. Six biochemicals showed extreme values that fall beyond at least five standard deviations from the mean, and one biochemical was absent from most

of the samples. These seven metabolite chemicals were excluded from further analysis because of concerns that these values reflect technical issues rather than biological effects, as per the experience of Metabolon. The four duplicate specimens included for quality control purposes (see section 2.1 Sample Collection and Metabolomics Profiling) were correctly clustered for each of the four participants, therefore we used the averaged metabolite readings of the duplicates as the readings for these four participants. Instrument variability (median RSD=7%) and total process variability (median RSD=8%) met the acceptance criteria.

3.1 Effects of Dental Decay and Family on Metabolite Profile

We estimated the Euclidean distance between all possible sample pairs based on the entire set of 275 metabolites. Metabolite profiles of the sibling pairs averaged a significantly smaller Euclidean distance compared to randomly selected participant pairs (Figure 1, Kolmogorov-Smirnov Test P -value=0.000324), suggesting that sibling pairs share metabolic function to a greater degree than expected by chance. The Euclidean distances between metabolite profiles of sibling pairs increased as the age difference between the participant pairs increased (Figure A.1.), although the percent of variance explained by age difference alone is low (Linear regression, R -squared=0.05).

The Euclidean distance between siblings with different dentition (primary and mixed) was significantly higher than between pairs where both siblings had primary dentition (Kolmogorov-Smirnov Test P -value = 6.699e-05). In addition to dentition, other factors may contribute to the variation of Euclidean distance. For instance, participant pairs who had larger differences in age appear to have larger Euclidean distances (Figure A.1.), but the low variance explained (r^2 =0.05) suggests that variation in Euclidean distance cannot be attributed solely to the age differences. The small sample size precludes determining the distribution of the correlation between Euclidean distance and age difference, which may not be linear. Because mixed dentition and age influence metabolite profile, the remaining analyses were limited to sibling pairs where both siblings have primary dentition.

The Euclidean distance between sibling pairs was smaller than the distance between randomly chosen pairs of participants from siblings matched by dental decay, lack of dental decay, or discordant by dental decay (Figure 2). Ignoring family relationships, the average pairwise Euclidean distance was smallest between children with dental decay, intermediate between children where one had decay and one did not, and greatest between children with sound teeth. Thus, the variability in metabolic profile among children with decay was much less than the variability among children with sound teeth.

3.2 Joint effects of familiarity and dental status

Using hierarchical clustering analysis, we observed a strong, statistically significant, effect of sibship on oral metabolite profile (Figure 3). This effect was especially strong for sibling pairs where both siblings had decayed teeth (Figure 3b). However, a similar effect was observed when both siblings had sound teeth (Figure 3a) and where siblings were discordant by decay status (Figure 3c). A test of the correlations within sibpairs for each biochemical using the Interclass Correlation Coefficient (ICC) found that among the 275 metabolites, 21 chemicals exhibited very high ICC values (>0.95) among sibpairs matched by dental decay, compared to the 7 chemicals in sibpairs matched by lack of dental decay, and 7 chemicals in sibpairs discordant by dental decay (Table 2).

We also compared the metabolite profile between participants with and without decay for all with children with primary teeth without controlling for sibling relationships. The

MANOVA analysis (via ADONIS package in R) showed no statistically significant difference between healthy and decayed samples (P-value=0.085, R-squared=0.080).

3.3 Do specific chemicals distinguish between healthy and decayed teeth?

In order to detect specific biochemical(s) that distinguish between healthy and decayed teeth, we used an ANOVA analysis with false discovery correction. This analysis suggested that a group of biochemicals involved in the arginine and proline pathway (aspartate, ornithine, arginine, proline) showed distinctive changes associated with dental decay (Figure A.2.). Using only these four molecules in a principle components analysis there was clear visual separation of samples collected from participants with and without dental decay (Figure 4). The ordination results also suggested familial effects on salivary metabolites, as samples from sibling pairs were closely distributed in the ordination space, especially sibling pairs matched on dental decay.

4.0 Discussion

We used an untargeted metabolomics screen of saliva to characterize the effects of familiarity, dentition and decay on oral metabolite profile among 25 sibling pairs. Sibling pairs represented four different combinations of dentition and dental decay status. We observed a strong effect of sibship, which may reflect the effects of common genes, environment and behaviors, and shared oral microbial communities. In our sample, using three different analyses (Euclidean distance, hierarchical clustering and PCA using selected biochemicals) metabolite profiles of saliva from children with decayed teeth were more similar than the metabolite profiles of saliva from children with healthy (sound) teeth. This suggests that the healthy oral state encompasses a wide range of physiological processes. The narrower range of metabolites from children with decay suggests that a subset of oral functions is affected by host-microbial interactions during dental decay.

The clustering analysis showed that the effect of decay on metabolic profile was largely nested within the stronger effect of sibship. If confirmed, this implies that functional biomarkers for dental decay across families may be hard to identify. However, our study provides some hints that some biomarkers might exist, as four molecules from the Arginine metabolism pathway changed with decay. While our sample size for this analysis is small and we cannot rule out that these findings are due to chance, our results are in agreement with previous studies, which report increased levels of free arginine and lysine in the saliva of caries-free individuals (VanWuyckhuysse et al., 1995; Vranic, 1991). Further, a 2009 study by Nascimento, et al., which compared the alkali generation of bacteria from caries free to caries active individuals, found bacteria from caries free individuals were more likely to produce ammonia from arginine (Nascimento et al., 2009). In 2012 Huang et al., using a dental polymicrobial biofilm model formed using saliva, found that sucrose hampered alkali production from arginine leading to lower pH and increased caries (Huang et al., 2012).

We did not find any previous reports in the literature describing untargeted whole sample salivary metabolomics in children with and without caries. A targeted salivary metabolite study among adults indicated that lipid and fatty acid profiles differed between caries free (n=22) and caries active adults (n=22) (Tomita et al., 2008). In a study of an unrelated oral disease, periodontitis, Liu et al. conducted whole sample untargeted metagenomics of plaque samples. Among adults with periodontitis (n=2) compared to healthy adults (n=3) there was greater redundancy in bacterial community composition, although the resulting oral functions were shared. Whether both oral function and oral bacterial community composition are shared in caries is an area of active research.

Saliva metabolites reflect ongoing functions of the oral microbiota and of the human host and may not be the optimal specimen for measuring metabolic responses to dental decay. Further, we have only a very limited understanding of the variability of salivary metabolite profiles. Thus, we do not know how sensitive these profiles are to behaviors such as using the same toothpaste or sharing a diet – as might be expected of siblings. However, saliva is easily collected, and at much larger volumes than plaque samples which would provide a more direct measure of dental decay. Further, if it is possible to identify biomarkers for decay in saliva – as this and other studies suggest (Guo, 2013; Tulunoglu et al., 2006) – it opens the door for the development of easily administered screening tests.

In conclusion, an untargeted metabolomics screen of saliva found a strong effect of sibship on the metabolite profiles identified; nested in the familial effects were associations of metabolite profile with dentition and with dental decay. The results of this pilot require confirmation in larger studies that include host behaviors, environmental factors, oral microbiota composition and structure, and host genetic predisposition are required to identify biomarkers for decay, and to estimate the relative contribution of host factors and oral microbes on risk of dental decay.

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Highlights

- We conducted an untargeted metabolomics screen of saliva.
- There was a strong effect of sibship on metabolite profile.
- Dentition and dental decay effects nested within familial effects
- Sibling effects reflect shared genetics, environment, behaviors

Figure 1.

Histogram of Euclidean distance between metabolite profiles of 25 sibling pairs. For comparison, the Euclidean distance for all possible pairings of the children is included. The distance between siblings (mean=13.6) was significantly smaller than that of all possible pairings (mean=18.0) (Kolmogorov-Smirnov Test P -value=0.000324). 50 children at high risk of dental decay, selected from participants in the Center for Oral Health in Appalachia (COHRA) study I.

Figure 2.

Histogram of Euclidean distances among 275 metabolites in untargeted metabolomics screen from 15 sibling pairs with primary dentition. Distances among all possible pairings regardless of family status ($n=435$), sib pairs ($n=15$); all possible pairs of children with decayed teeth ($n= 91$ pairs); all possible pairs of children with sound teeth ($n= 120$ pairs); and all possible pairings of children with decayed and sound teeth ($n= 224$ pairs). 30 children with primary dentition at high risk of dental decay, selected from participants in the Center for Oral Health in Appalachia (COHRA) study I.

Figure 3.

Hierarchical clustering analysis by sibpairs groupings chosen to represent combinations of decay status among children with primary teeth. The dissimilarity between samples was estimated using the correlation method, indicated on the y-axis. Each participant's family is numbered following "Fam". In (c), the participant's decay status and dentition is indicated in addition to the family number. Red numbers are the Approximately Unbiased P -value (ranging 0 to 1) of its adjacent cluster, calculated via multiscale bootstrap resampling. Higher P -values indicate clusters are more strongly supported by data. Rectangles with solid line indicate correctly clustered sib pairs that are strongly supported by data (P -value > 0.9); rectangles with dotted line indicate correctly clustered sib pairs, although the confidence from data is lower (P -value < 0.9). 30 children with primary dentition at high risk of dental decay, selected from participants in the Center for Oral Health in Appalachia (COHRA) study I.

Figure 4.

Principle Correlation Analysis using Aspartate, Ornithine, Arginine and Proline showing the separation of participants with sound and decayed teeth, as indicated using the 95% dispersion ellipses of participants with (dotted line) and without decay (solid line) along PC1 and PC2. Only samples from sibling pairs where both children have primary teeth are included. Samples from the same families (indicated by the numbers in each data point) are closer together suggesting host-bacterial interactions of the biochemicals in sibling pairs from the same families. Sibling pairs where both children have decayed teeth are distributed especially close to each other. 30 children with primary teeth at high risk of dental decay, selected from participants in the Center for Oral Health in Appalachia (COHRA) study I.

Characteristics of 25 sibling pairs at high risk of dental decay, selected from children participating in the Center for Oral Health in Appalachia (COHRA) study I. The Decayed, Missing and Filled Teeth index (DMFT) is commonly used for dental decay, and is calculated as number of teeth with active decays + missing due to decays + filled teeth.

Table 1

Dentition Sib1/Sib 2	Tooth Health Sib1/Sib 2	Number of pairs	Mean Age	Mean Age Difference	Mean Teeth#	Mean DMFT
Primary/Primary	Sound/Sound	5	3.4	2	18.6	0
Primary/Primary	Sound/Decayed	6	3.1	2.5	16.9	1.8
Primary/Primary	Decayed/Decayed	4	3.1	1.2	18.9	6.2
Primary/Mixed	Sound/Sound	10	4.9	4.8	18.6	0

Table 2

Super pathway, sub pathway and biochemical name for 21 biochemicals that exhibited >0.95 ICC value. Results of an untargeted metabolomics screen of saliva from 50 children at high risk of dental decay, selected from participants in the Center for Oral Health in Appalachia (COHRA) study I.

	Super Pathway	Sub Pathway	Biochemical Name
1	Amino acid	Histidine metabolism	Trans-uocanate
2	Amino acid	Phenylalanine & tyrosine metabolism	3-(4-hydroxyphenyl) lactate
3	Amino acid	Valine, leucine and isoleucine metabolism	Valine
4	Amino acid	Creatine metabolism	Creatine
5	Amino acid	Glutathione metabolism	5-oxoproline
6	Peptide	Dipeptide	Glycylvaline
7	Peptide	Dipeptide	Glycylphenylalanine
8	Peptide	Dipeptide	Alanylleucine
9	Peptide	Dipeptide	Alanyllysine
10	Peptide	Dipeptide	Arginyltyrosine
11	Peptide	Dipeptide	Aspartylleucine
12	Peptide	Dipeptide	Histidylleucine
13	Peptide	Dipeptide	Isoleucyltyrosine
14	Peptide	Dipeptide	Leucylalanine
15	Peptide	Dipeptide	Arginylmethionine
16	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism	Lactate
17	Energy	Krebs cycle	Citrate
18	Lipid	Lysolipid	1-palmitoleoylglycerophosphoethanolamine
19	Lipid	Lysolipid	1-linoleoylglycerophosphoethanolamine
20	Lipid	Lysolipid	1-palmitoylglycerophosphocholine
21	Lipid	Monoacylglycerol	1-palmitoylglycerol (1-monopalmitin)