# Genomic insights into childhood oral health and disease: what we know & what is coming

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## THE WEB OF KNOWLEDGE ON ORAL HEALTH AND DISEASE IN EARLY CHILDHOOD

Concerted efforts during the last few decades have brought about a comprehensive understanding of the proximal and distal determinants of common oral diseases in children, including their fundamental causes. The logical next step following the major advances in the science and practice of dentistry is the translation of this new knowledge to better oral health care of individuals and populations—providing optimal, customized oral health care according to individuals' specific needs is an ambitious but achievable goal, now termed "precision dentistry". Nevertheless, substantial gaps still need to be addressed in both our evidence base [Mejàre et al., 2015] and the translational process [Casamassimo et al., 2014].

Dental caries is the most common chronic childhood disease, affecting approximately 25% of children in most populations. Current concepts depict it as a multifactorial disease caused in a dysbiotic oral microbial environment in which fermentable dietary carbohydrates are metabolized producing acidic byproducts that dissolve enamel in susceptible teeth. This process quickly destroys the thin enamel of primary teeth, creating clinical cavities, dental infection, and tooth loss. Early childhood caries (ECC) is an early-onset aggressive form of the disease that frequently requires restorative care under sedation or general anesthesia. ECC confers substantial human and economic costs on children, their families, and the public health care infrastructure [Casamassimo et al., 2009]. The disease burden falls disproportionately on minority or socioeconomically disadvantaged families, whose children have more than twice the risk of developing ECC as their non-poor counterparts, and who are frequently unable to access necessary preventive and restorative dental care. Despite a general decrease in caries prevalence among older children and adults, the proportion of preschool children with ECC has persisted and possibly increased among population subgroups. This suggests that public health- and personal-preventive care, while effective in preventing caries in older children, has had little impact on ECC. An explanation for the persistence of this early-onset, aggressive form of caries in young children therefore must look beyond established behavioral, environmental, and societal risk factors.

## THE CASE FOR GENETICS

The overwhelming dominance of social determinants and upstream factors in children's oral health outcomes is well-documented [Lee and Divaris, 2014]. While much known about behavioral mediators such as diet, fluoride exposure, oral hygiene and dental care [Garcia et al., 2015] a substantial genetic basis of dental caries has long been theorized. A genetic component of dental caries was suggested by several twin studies [Goodman et al., 1959; Horowitz et al., 1958] which collectively included 609 pairs of monozygotic and 569 pairs of dizygotic twins, providing strong evidence of a genetic contribution to caries risk. Reported heritability estimates for dental caries range between 30% and 76% and are higher for primary dentition

**Table 1.** Phenotypic variance explained for early childhood caries (ECC) case status and severity ($d_1d_{2,3}$mfs index index) among the 212 3 and 4-year-old children enrolled in the ZOE-G4S study.

| | ECC case status (binary case definition) | | ECC severity ($d_1d_{2,3}$mfs index) | |
|---|---|---|---|---|
| | Variance explained (standard error) | Likelihood ratio p-value | Variance explained (standard error) | Likelihood ratio p-value |
| **ALL SNPS (n= 2331188)** | | | | |
| Only SNPs considered | 0.43 (0.36) | 0.043 | 0.06 (0.08) | 0.12 |
| +Age, Sex | 0.38 (0.36) | 0.07 | 0.06 (0.08) | 0.13 |
| **MAF>0.01 (n= 1877037)** | | | | |
| Only SNPs considered | 0.44 (0.39) | 0.034 | 0.07 (0.08) | 0.08 |
| +Age, Sex | 0.39 (0.39) | 0.061 | 0.07 (0.08) | 0.08 |
| **MAF>0.05 (n= 1382931)** | | | | |
| Only SNPs considered | 0.52 (0.55) | 0.026 | 0.14 (0.14) | 0.01 |
| +Age, Sex | 0.43 (0.50) | 0.050 | 0.13 (0.14) | 0.02 |
| **MAF > 0.10 (n=986805)** | | | | |
| Only SNPs considered | 0.48 (0.53) | 0.026 | 0.21 (0.20) | 0.006 |
| +Age, Sex | 0.39 (0.48) | 0.050 | 0.19 (0.19) | 0.008 |
| MAF, minor allele frequency | | | | |

versus permanent dentition caries [Dawson, 2008; Schuler, 2001; Shaffer et al., 2012]. Noteworthy, Bretz and colleagues [Bretz et al., 2006] found that genetic susceptibility to caries is independent of the heritability of sweetness preference. These estimates range between 40 and 60% for ECC, whereas heritability in our own preliminary studies [Ballantine et al., unpublished observations] among 3 and 4 year-old children is 44% (Table 1).

We now know that genetics in general, is a risk factor for ECC; however, we know next to nothing about specific, ECC risk-conferring genetic influences. Acknowledging the importance of genetics, the first U.S. National Institutes of Health-sponsored Consensus Development Conference on dental caries included in its major directions for clinical caries research, "genetic studies are necessary to identify genes and genetic markers of diagnostic, prognostic, and therapeutic value". Most evidence to-date has emanated from candidate-gene studies, which have investigated a number of genes 'plausibly implicated' in caries development. Results of this body of literature have provided some initial leads that genes involved in sweet taste preference [Kulkarni et al., 2013], enamel formation [Shimizu et al., 2012], and host immunity [Brise-o-Ruiz et al., 2013] may be associated with caries risk. However, the inherent limitations of the candidate-gene approach diminish the inferential potential of this evidence base, especially in light of data starting to emanate from genome-wide association (GWAS) and whole genome sequencing studies (WGS).

## NEXT-GENERATION APPROACHES: GENOME-WIDE ASSOCIATION

During the last 10 years, GWAS have revolutionized the study of genetic contributions to human health providing an unbiased approach to assess complex traits including obesity and type II diabetes [Hirschhorn et al., 2005; Wang et al., 2005; McCarthy et al., 2008]. GWAS capitalize on technological advances that permit the efficient interrogation of millions of nucleotide polymorphisms and genetic loci in an agnostic manner, without limited by prior knowledge or hypothesized biological plausibility. Because there is strong evidence suggesting the genetic susceptibility to caries, a GWAS is the next logical step in the unbiased interrogation of common genetic variation associated with ECC. The completion of the one thousand genomes [1000 Genomes Project Consortium, 2012] and ENCODE [ENCODE Project Consortium, 2012] projects add further value in the characterization and annotation of GWAS results, via their linkage with known functional elements of the human genome.

Still today, GWAS are rare in the oral health domain, with a handful of investigations carried out for periodontitis [Divaris et al., 2013; Teumer et al., 2013; Shaffer et al., 2014] and even less for dental caries. A recent multi-cohort GWAS among 7,400 European American (EA) adults [Wang et al., 2012] found no genome-wide significant signals for dental caries, presumably due to the pronounced heterogeneity in participants' ages, dental caries assessment methods, genotyping platforms, and study designs. The one GWAS to-date of primary dentition caries involved 1,300 EA children aged 3-12 [Shaffer et al., 2011]. It found suggestive evidence of association for 7 loci two of which (*MPPED2* and *ACTN2*) showed statistically significant gene-centric associations in a follow-up study that included additional independent child and adult samples [Stanley et al., 2014]. Of note, these findings were derived from predominantly EA populations, with only 104 African American and no Hispanic/Latino or Asian children. Moreover, this study included children predominantly over the age of 6, a time when primary teeth start to shed, leading to obvious underestimation of ECC. In sum, our current understanding of the genomic basis of ECC heritability is categorically limited and improvements on sampled populations, phenotype characterization, and genomic methods are warranted.

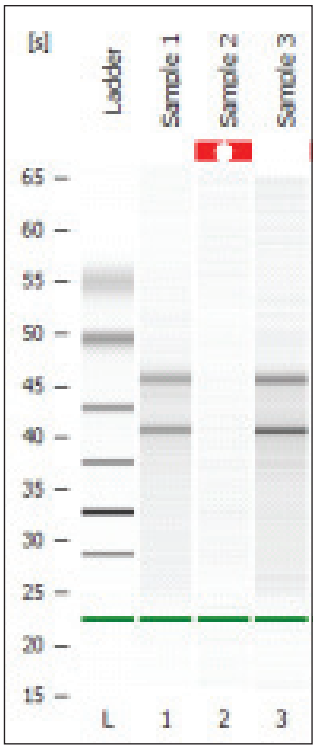


**Figure 1.** Agilent assay

## PRELIMINARY EVIDENCE: THE ZOE-G4S STUDY

In 2013, our University of North Carolina-Chapel Hill group embarked on the goal to comprehensively characterize the genomic basis of ECC, and in a second step provide insights into ECC-defining metagenomics, metatranscriptomics, proteomics and metabolomics events at the tooth surface-biofilm interface. The Zero-out Early Childhood Caries- Genes for Smiles (ZOE-G4S) study is a 5-year project (2015-2010) focused on conducting a large-scale GWAS of ECC among a planned multi-ethnic sample of approximately 9,000 3 to 5-year-old children attending preschool centers in North Carolina (NC). To-date, we have enrolled and examined approximately 750 children and comprehensive genotyping involving 2.5 million single nucleotide polymorphisms (SNPs) has been carried out among 212 of those.

## CLINICAL EXAMINATION AND BIOSPECIMEN COLLECTION

The clinical examination protocol involves comprehensive dental examinations for dental caries (tooth surface-level diagnoses using ICDAS visual criteria), hypoplastic defects of the enamel, dental trauma, occlusal characteristics,

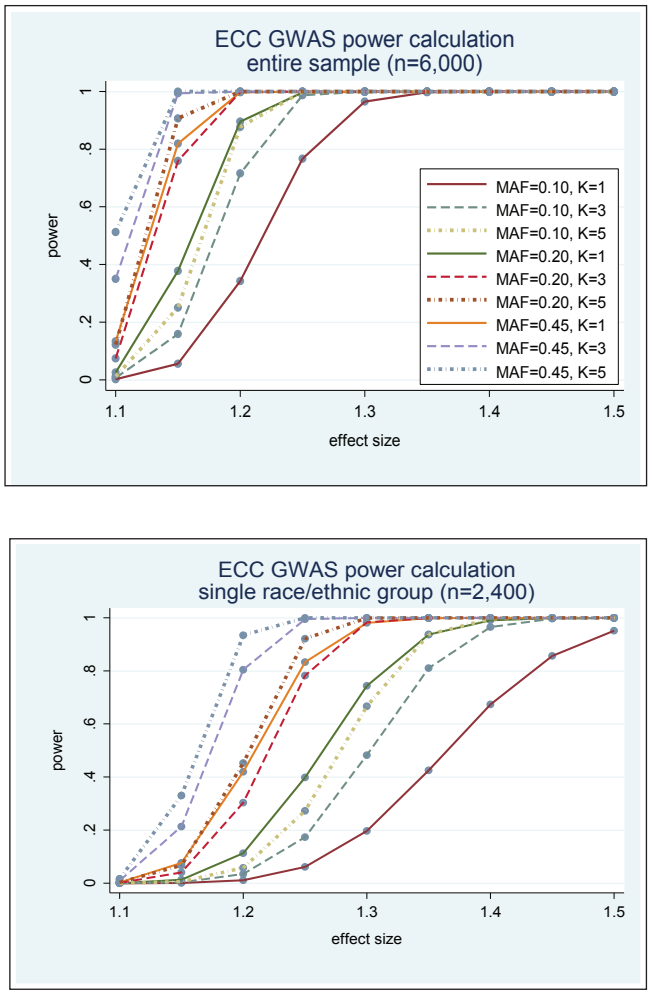


**Figure 2.** GWAS Power calculations for (K) 1, 3, and 5 discoverable SNPs.

as well as body mass index. In an assessment of intra-examiner reliability that we carried using duplicate exams of 23 children, surface-specific caries classification showed excellent reliability (kappa= 0.85, 95% CI=0.83-0.88). Before recording caries, the examiner brushes the teeth to remove dental plaque and surface-level caries examinations are done using artificial light, magnification, and compressed air. Children are given the toothbrush and toothpaste, along with oral hygiene instruction and a 'wellness report card' with significant findings and pertinent information for his or her family.

Saliva samples are collected using an "assisted donation" method using the DNA Genotek Oragene-575 kit. DNA is subsequently purified using a Perkin-Elmer Magnetic bead-based MSMI extraction robot. Overall, we have been obtaining DNA of sufficient yield and good quality for genotyping (in μg): Optical Density=42.3 (SD=24.6), Picogreen=28.7 (SD=15.2), and human-specific DNA concentration=4.0 (SD=1.6); and >80% with A260/A280 ratio between 1.6 and 2.0. Genotyping has so far being done using the HumanOmni2.5-8 array, offering ~2.5 million markers, with excellent results among the first 212 samples [Barakat et al., 2015]: no sex mismatches, median (range)—sample call rate=99.90% (96.12%-99.96%) and replicate error rate=$6x10^{-7}$ ($2x10^{-6}$ - $4x10^{-7}$).

For the collection of supragingival microbial plaque from buccal and interproximal surfaces of pre-determined index teeth we have used sterile wooden tooth picks. If index teeth are missing, we sample contralateral teeth instead, followed by ipsilateral. The plaque samples are pooled by dispersal in RNAlater Tissue Protect tubes (QIAGEN) transferred within 24 hours to the UNC BioSpecimen Core processing facility, where they currently stored at -80°C until further processing. Microbial DNA and total RNA are extracted from plaque samples using the Duran-Pinedo protocol [Duran-Pinedo et al., 2014] using the mirVana isolation kit (Life Technologies) for RNA and the ToTALLY RNA (Life Technologies) for DNA. Data obtained so far indicate good yield (~300ng) and good integrity RNA as determined by Agilent assays (samples 1 and 3 in Figure 1). Subsequently, microbiome analyses commence with library preparation and sequencing for 16S rRNA for taxonomy and mRNA/cDNA for metatranscriptome. Analyses of 16S amplicon-sequencing data are carried out using the QIIME pipeline, whereas the cDNA library pool is sequenced on the Illumina HiSeq platform. Bioinformatics pipelines are used to remove human sequences and align bacterial ones to genomes obtained from the Human Oral Microbial Database.

## PRELIMINARY HERITABILITY ANALYSES

We have used the directly genotyped (non-imputed) set GWAS data and Visscher's GCTA approach [Yang et al., 2013] to estimate the phenotypic variance explained by all 2.5 million SNPs in the first sample of 212 genotyped children. We found 44% (se=0.39; P=0.03) heritable variance in the ECC binary case definition explained by all non-rare (minor allele frequency>0.1) GWAS SNPs, an estimate that increased when we consider more common SNPs (Table 1). Adjusting for age and sex somewhat reduced the heritable variance explained and further reductions are expected after formal adjustments for population stratification via EIGENSTRAT [Price et al., 2006] principal component analysis. This is particularly important for a multi-racial cohort like the present one, wherein the racial/

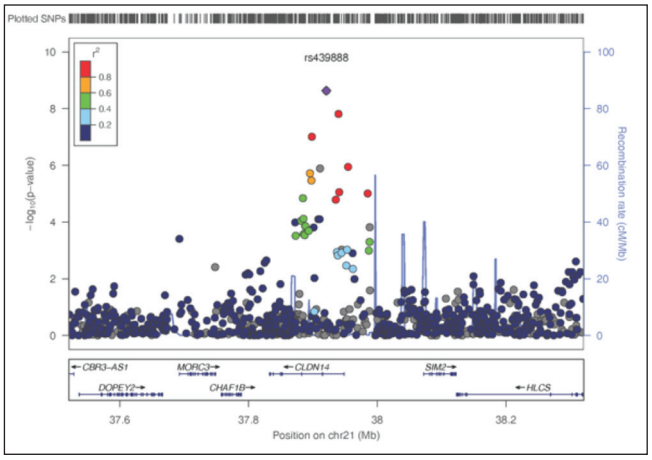


**Figure 3.** The CLDN14 locus highlighted for the association between rs43988 with the ECC binary definition ($p=2.3x10^{-9}$).

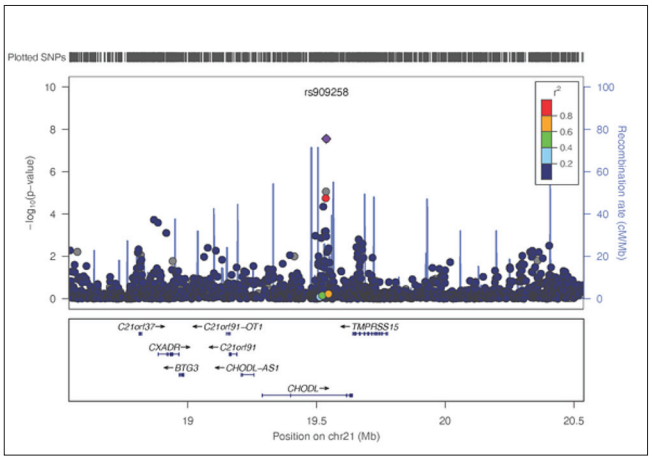


**Figure 4.** The CHODL locus highlighted for the association between rs909258 with the dmfs index ($p=2.8x10^{-9}$).

ethnic distribution so far is: Hispanic/Latino=36%, African-American=31%, European-American=23%, Native American and "other'=9%. As expected, less variance (between 6% and 21%) was explained by the GWAS SNPs in the dmfs index; the latter is known to be affected among other factors by the receipt of dental care. Considering that these estimates are the lowest limit of true heritability and the very small pilot sample size of just over two hundred, one can be confident that a well-powered, multi-ethnic GWAS among a large population is well-justified and will be fruitful.

## SINGLE MARKER DISCOVERY GWAS

The GWAS methodology is subject to a high rate of false positives rate due to the simultaneous testing of millions of markers via logistic regression (for the ECC binary case definition) or linear regression (for the dmfs index) genetic models. For this reason, a Bonferroni multiple-testing correction assuming 1 million independent tests is conventionally assumed, resulting in a genome-wide statistical significance threshold of p<5x10-8. The implications of using this conservative cut-off include on the one hand a protection against false positives and a high probability of missing true 'good signals' that happen to lie below that stringent significance criterion on the other [Shi et al., 2011]. Another obvious consequence of testing millions of markers is that sample sizes in the range of thousands are required to obtain sufficient power to detect genome areas (loci) of even moderate effect. As shown in Figure 2, the study has modest power to detect small effects (odds ratio between 1.2 and 1.3) even with a projected sample size of 6,000 (top panel), while power decreases further when racial/ethnic-stratified analyses are considered. The initial genome-wide scans revealed two loci in chromosome 21 that met the p<5x10-8 statistical significance threshold, one for the ECC binary trait and another for the dmfs index. These are shown in Locus Zoom plots in Figures 3 and 4. Claudin 14 (CLDN14) encodes a protein that is an integral membrane protein and a component of tight junction strands. Defects in this gene are the cause of an autosomal recessive form of non-syndromic sensorineural deafness. It has also been reported that four synonymous variants in this gene are associated with kidney stones and reduced bone mineral density. On the other hand, the chondrolectin (CHODL) gene encodes a type I membrane protein with a carbohydrate recognition domain characteristic of C-type lectins in its extracellular portion.

## SUMMARY OF FINDINGS AND NEXT STEPS

These early results provide primarily evidence on the feasibility of the followed approach: obtaining high-quality genotypes, as well as microbial plaque DNA and RNA, via the examination of a community-based (non-clinical) sample of preschool children under field conditions. Using these data we are able to gain initial insights into specific loci that may be underlying ECC risk (as a binary trait) and severity (as a continuous trait) and for the first time, obtain heritability estimates for ECC. These findings will need to be replicated in the larger planned sample of approximately 6,000 children, as well as other independent cohorts of children and potentially adults. As shown in Figure 5, two genome-wide significant loci have been identified for dental caries among the European-American sample (n=4,504) of the

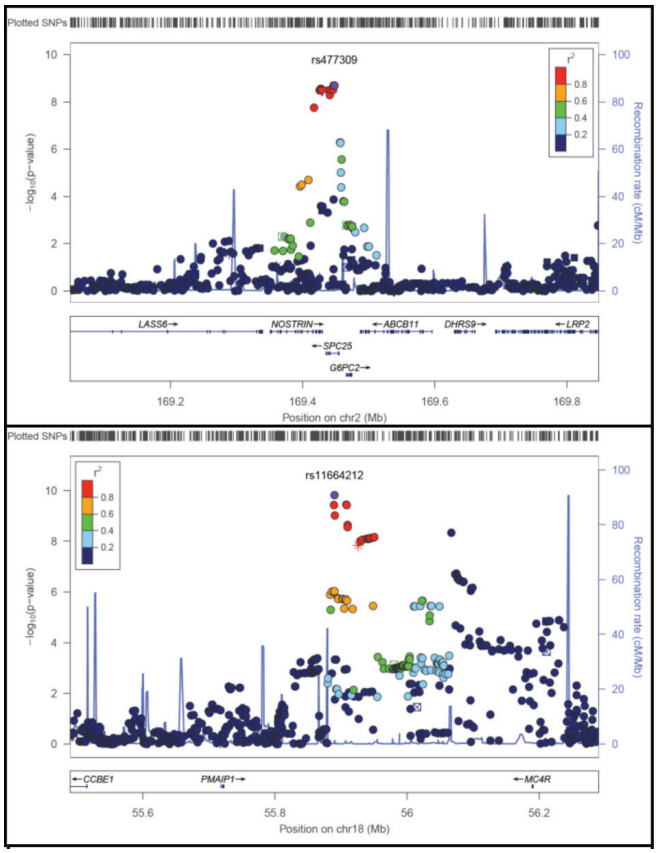


**Figure 5.** Visualization of two genome-wide significant loci for the DMFS index in the European-American adult cohort of the Atherosclerosis Risk in Communities study.

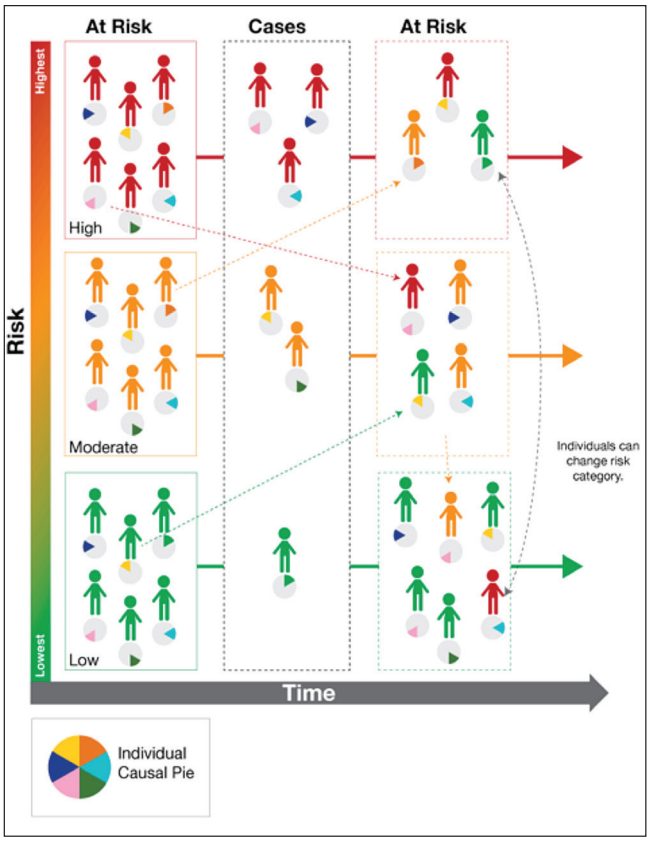


**Figure 6.** Schematic illustration of ECC risk ascribed to population groups along with individual heterogeneity depicted by person-level susceptibility factor sets ("causal pies"). Achievement of precision dentistry is based on the identification of heterogeneous individual susceptibility factors including host genome and oral microbiome, above and beyond population-level influences and potentially modifiable behaviors.

Atherosclerosis Risk in Communities study [The ARIC Investigators, 1989]. Evidence on loci identified by GWAS for dental caries among children and adults must be validated with prospective, experimental and mechanistic studies, and ultimately evaluated as prognostic markers chairside. The superimposition of metagenomic and metatranscriptomic data in a systems biology framework will further augment the biological and clinical relevance of genomic risk factors [Nyvad et al., 2013].

## CLINICAL AND PUBLIC HEALTH RELEVANCE

Unraveling the genetic underpinnings of common, complex diseases like ECC has the potential to benefit clinical care and lead to improvements in population health. By identifying biologically informative genetic variation in ECC, one can reasonably expect to shed new light on the etiology of the disease, potentially contributing to interventions for prevention or treatment and new strategies for risk stratification to target children most likely to develop ECC. Although in 2015 modifying human genetic variation is still impractical, knowledge of specific genetic contribution and mechanistic pathways underlying ECC can aid in and inform discoveries in chemotherapeutics, probiotics, biologicals, drugs, dental materials and other domains, that can change the medical management of the disease.

Finally, it is important to acknowledge that the paradigm of precision dentistry goes beyond the identification of "high risk" groups, which are known to be driven by population characteristics and influenced by social determinants of health. Figure 6 illustrates the fundamental difference between considering ECC risk (a population-level attribute) [Rose, 1985] and individual susceptibility [Vineis

and Kriebel, 2006], as depicted by person-level susceptibility factor sets ("causal pies"). Achievement of precision dentistry is exactly based on the identification of the heterogeneous and largely unobservable individual susceptibility factors including host genome and oral microbiome, above and beyond population-level influences and potentially modifiable behaviors. Concerted efforts by all stakeholders will be required so that these advances benefit populations in an equitable manner; it is an ethical imperative that those most in need receive the most preventive and therapeutic benefits.

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