Association Between Nitrate-Reducing Oral Bacteria and Cardiometabolic Outcomes: Results From ORIGINS

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Abstract

Background

The enterosalivary nitrate-nitrite-nitric oxide pathway is an alternative pathway of nitric oxide generation, potentially linking the oral microbiome to insulin resistance and blood pressure (BP). We hypothesized that increased abundance of nitrate-reducing oral bacteria would be associated with lower levels of cardiometabolic risk cross-sectionally.

Methods and Results

ORIGINS (Oral Infections, Glucose Intolerance, and Insulin Resistance Study) enrolled 300 diabetes mellitus–free adults aged 20 to 55 years (mean=34±10 years) (78% women). Microbial DNA was extracted from subgingival dental plaque (n=281) and V3–V4 regions of the 16S rRNA gene were sequenced to measure the relative abundances of 20 a priori–selected taxa with nitrate-reducing capacity. Standardized scores of each taxon's relative abundance were summed, producing a nitrate-reducing taxa summary score (NO_3 TSS) for each participant. Natural log-transformed homeostatic model assessment of insulin resistance, plasma glucose, systolic BP, and diastolic BP were regressed on NO_3 TSS in multivariable linear regressions; prediabetes mellitus and hypertension prevalence were regressed on NO_3 TSS using modified Poisson regression models. Nitrate-reducing bacterial species represented 20±16% of all measured taxa. After multivariable adjustment, a 1-SD increase in NO_3 TSS, was associated with a −0.09 (95% CI, −0.15 to −0.03) and −1.03 mg/dL (95% CI, −1.903 to −0.16) lower natural log-transformed homeostatic model assessment of insulin resistance and plasma glucose, respectively. NO_3 TSS was associated with systolic BP only among patients without hypertension; 1-SD increase in NO_3 TSS was associated with −1.53 (95% CI, −2.82 to −0.24) mm Hg lower mean systolic BP. No associations were observed with prediabetes mellitus and hypertension.

Conclusions

A higher relative abundance of oral nitrate-reducing bacteria was associated with lower insulin resistance and plasma glucose in the full cohort and with mean systolic BP in participants with normotension.

Keywords: epidemiology, high blood pressure, insulin resistance, nitrate, oral microbiome

Subject Categories: Epidemiology, Risk Factors, Diabetes, Type 2, High Blood Pressure, Hypertension

Clinical Perspective

What Is New?

- The oral microbiome plays an important role in the enterosalivary nitrate-nitrite-nitric oxide pathway.
- To the best of our knowledge, this is the first study to directly examine the relationship between specific nitrate-reducing oral microbiota and cardiometabolic outcomes in a population setting.
- Our results support the hypothesis that oral nitrate-reducing bacteria play a beneficial role in blood pressure regulation and insulin resistance.

What Are the Clinical Implications?

- If this relationship proves to be causal, oral microbial risk factors for cardiometabolic outcomes may be identified, and further research could yield useful treatments that manipulate the oral microbiome to improve cardiometabolic health.

Introduction

Increasing evidence suggests that the digestive tract microbiome (ie, bacteria colonizing the oral cavity and the gastrointestinal tract) may contribute to the development of insulin resistance,[1, 2] type 2 diabetes mellitus,[3, 4] and hypertension.[5, 6] These associations between the oral microbiome and increased cardiometabolic risk are most commonly hypothesized to result from a chronic inflammatory response to a dysbiotic subgingival microbiome.[7] However, a possible alternative mechanism is via the production of the physiologically important gaseous transmitter, nitric oxide (NO).

NO is an important signaling molecule involved in many physiological processes, including endothelial function, vasodilation, immune function, glucose metabolism, and blood pressure (BP) control.[8] A loss of NO production and bioavailability has been implicated in the pathogenesis of insulin resistance and hypertension.[9, 10] NO production was originally thought to occur solely through the endogenous conversion of L-arginine and oxygen into NO and L-citrulline by NO synthases found in the endothelium and other tissues. However, it has recently been discovered that NO production can also occur via the reduction of salivary nitrates by nitrate-reducing oral bacteria to form nitrites, which are then swallowed and made systemically bioavailable for further reduction into NO in the blood vessels and tissues.[8] This so-called enterosalivary nitrate-nitrite-NO pathway presents a novel and biologically plausible mechanism by which oral bacteria might influence the systemic bioavailability of NO and the development of related clinical cardiometabolic outcomes in humans.

The enterosalivary pathway is thought to underlie the strong evidence from experimental studies suggesting that increased dietary nitrate intake has beneficial systemic cardiometabolic effects. A systematic review of 13 trials lasting 1 to 6 weeks found an ≈4.1 and 2.0 mm Hg reduction in systolic BP (SBP) and diastolic BP (DBP), respectively, following daily nitrate supplementation.[11] Studies in mice have shown that dietary nitrate can improve insulin signaling and reverse features of metabolic syndrome.[12, 13] Reduced plasma glucose and improved insulin sensitivity following nitrate supplementation have also been observed in some human studies, although the results are less conclusive.[14, 15, 16] The direct role of nitrate-reducing oral bacteria in the enterosalivary pathway of NO production is supported by several small experimental studies that use antibacterial mouthwash to reduce the overall oral bacteria. Antibacterial mouthwash use significantly blunts the BP and plasma glucose reductions observed following experimental nitrate supplementation.[14, 17, 18] Notably, even in the absence of exogenous nitrate supplementation, a decrease in salivary and plasma nitrite, and an ≈3 mm Hg increase in SBP and DBP was observed after antibacterial mouthwash use.[19] This finding suggests that oral microbiota play a continuous role in BP regulation through the nitrate-nitrite-NO pathway.

To our knowledge, no study has directly investigated the relationship between specific oral microbiota with known nitrate-reducing capacity and cardiometabolic outcomes in a population setting. Only a few clinical trials have directly correlated abundance of nitrate-reducing oral bacteria with cardiometabolic outcomes[20, 21] and associations for only a few species of nitrate-reducing bacteria were reported. Furthermore, the population distribution of oral nitrate-reducing bacteria remains unexplored and it is unknown whether nuanced variation, rather than the extreme differences in nitrate-reducing taxa created by mouthwash use, is beneficially related to cardiometabolic parameters. The purpose of this study is to examine the cross-sectional relationship between subgingival nitrate-reducing bacteria and cardiometabolic outcomes in diabetes mellitus–free adults enrolled in ORIGINS (Oral Infections, Glucose Intolerance, and Insulin Resistance Study).[4] We hypothesize that higher relative abundance of nitrate-reducing oral bacteria will be associated with lower levels of insulin resistance and BP, as well as a lower prevalence of prediabetes mellitus and hypertension.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Description of ORIGINS

ORIGINS is a cohort study investigating the relationship between subgingival microbial community composition and impaired glucose metabolism.[4] The cross-sectional data used in this study are from the baseline wave 1 (n=300 participants) enrolled from February 2011 to 2013, with the following inclusion criteria: (1) aged 20 to 55 years; (2) no diabetes mellitus (type 1 or type 2) based on participant self-report, glycated hemoglobin values <6.5%, and fasting plasma glucose <126 mg/dL; and (3) no self-reported history of myocardial infarction, congestive heart failure, stroke, or chronic inflammatory conditions. All participants reported not taking antibiotics in the past 30 days. Participants underwent oral examinations (including periodontal measurements), collection of oral bacteria specimens, blood draw after an overnight fast, and in-person anthropometric assessments at the same visit. Columbia University's institutional review board approved the study protocol. All participants provided written informed consent.

Of the 300 participants, the present analyses include only the 281 participants without missing 16S rRNA data or important baseline

cardiometabolic risk factors.

Bacterial Assessment and Identification

Subgingival plaque samples (n=281) were collected from prespecified sites. The mesiobuccal site of the second-most posterior tooth in the lower left quadrant (excluding third molars) of each participant was sampled using sterile curettes after removal of the supragingival plaque.[4] The samples were suspended in 300 µL of TE buffer (50 mmol/L Tris, 1 1 mmol/L EDTA; pH 7.6), and microbial DNA was extracted using the MasterPure Gram Positive DNA Purification Kit (Epicentre).[22]

Next-generation sequencing of the 16S rRNA gene was performed at the Forsyth Institute. The Human Oral Microbiome Identification using Next Generation Sequencing (HOMINGS) methodology[22, 23] is designed specifically for oral taxa, generating species-level information with high precision. Briefly, 50 ng of DNA was used to amplify the V3–V4 region of the 16S rRNA gene (using 341F/806R universal primers) and PCR products were purified using AMPure beads. Amplicons were then sequenced on the MiSeq (Illumina) platform. Paired-end reads were joined using QIIME join fastq; minimum overlap was set to 70 bp, and the percent max difference was 25%. Nonbarcoded sequences and sequences with a Phred quality score <25 were excluded. Samples with <5000 reads were excluded from the analyses. Overall, 18 531 931 sequences were generated for final analysis (median of 75 977 sequences per sample).

HOMINGS follows an in silico hybridization process. A BLAST program, called "ProbeSeq for HOMINGS," uses specially designed in silico species-specific 16S rRNA-based oligonucleotide probes to identify species taxa and frequency.[24] An array is created using the raw sequence files and the program loops through examining one sequence at a time, looking for a "string" that fully matches one of the probes. The total number of matches, or unique in silico-hybridization events, are then counted with each match representing the conceptual identification of 1 bacterial cell. ProbeSeq is an iterative process, and sequences not detected by a species-level probe are then processed against genus-level probes. The final HOMINGS data output for each individual are expressed as the relative abundance of each target taxa (by dividing the respective HOMINGS hits for that taxa by the sum of all taxa hits within the individual, ie, percent proportions of each target taxa). Overall, each sample had an average of 22% (SD=12%) unmapped reads that matched to neither species nor genus probe. Using HOMINGS, 668 different taxa were identified in ORIGINS, with an average of 182 (SD=50) taxa identified in each participant sample.

Operationalization of Nitrate-Reducing Oral Bacteria Exposure

Exposure to nitrate-reducing oral bacteria was defined by creating a summary score comprising oral bacteria species previously identified in the literature as being associated with nitrate-reduction capacity.[25, 26] From the list of 28 putative nitrate-reducing oral species (Table S1), 20 taxa were identified by HOMINGS.[27] These 20 nitrate-reducing bacteria overall showed low correlations with each other (Figure S1). To address the skewed distributional properties of using proportional data, a variance-stabilizing arcsin-square root transformation commonly used in microbiome analyses was first applied to the relative abundance of each taxa.[28] The arcsin-square root transformed relative abundance of each taxa was then standardized by dividing by its SD, as per an a priori approach described elsewhere.[4] Standardized values for each of the nitrate-reducing taxa were then summed, creating a summary score representing the total nitrate-reducing microbiota community exposure in the sample. The standardization gives equal weight to each taxa, and, without complete knowledge of their nitrate-reducing capacity, prevents a summary score from being dominated by the most abundant taxa (Table S2).

Outcomes

Insulin resistance and plasma glucose

Plasma glucose and insulin levels were measured from blood collected following an overnight fast. Insulin resistance was measured using homeostatic model assessment of insulin resistance (HOMA-IR) values calculated from fasting insulin and glucose levels.[29]

Prediabetes Mellitus

Prediabetes mellitus (yes/no) was defined in accordance with the American Diabetes Association criteria as follows: (1) fasting plasma glucose ≥100 mg/dL and <126 mg/dL; or (2) glycated hemoglobin ≥5.7% and <6.5%.[30]

SBP and DBP

Seated resting SBP and DBP were measured in triplicate and the last 2 measurements averaged to obtain our continuous measures of mean SBP and DBP (mm Hg).

Hypertension

Hypertension (yes/no) was defined in accordance with the most recent 2017 American Heart Association criteria as

follows: (1) an SBP recording of ≥130 mm Hg; or (2) a DBP recording ≥80 mm Hg.31 Participants were also classified as having hypertension if a diagnosis of hypertension was self-reported.

Risk Factor Assessment

Cardiometabolic risk factors were measured by trained research assistants as previously described.4, 32 Participant body mass index was calculated as weight in kilograms/height in meters.2 Questionnaires were administered to obtain information on: age, sex, race/ethnicity (non-Hispanic black, non-Hispanic white, Hispanic, other), educational level (high school completion, college/vocational training, advanced degrees), cigarette smoking (current, former, or never smoking, and duration/intensity of smoking). Overall dietary pattern was assessed using the Alternative Healthy Eating Index 2010 (AHEI 2010) that was created based on foods and nutrients predictive of chronic disease risk.33 The index consists of several components: vegetables, fruits, whole grains, nuts and vegetable protein, red/processed meat, sugar-sweetened beverages and fruit juice, trans fats, polyunsaturated fats, long-chain fatty acids, sodium, and alcohol consumption. Each food group has a range of 0 to 10 points, which are then summed to create the overall score. The AHEI 2010 score ranges from 0 to 110, with higher AHEI scores associated with a lower risk of coronary heart disease and diabetes mellitus.33 Leisure-time physical activity was assessed and converted to metabolic equivalents, and participants were categorized into 4 leisure-time physical activity categories as previously described.32 Measures of periodontitis were obtained from the clinical periodontal examinations as previously published,4 and periodontal status was measured by the percentage of periodontal sites with attachment loss ≥3 mm. (see Data S1 for additional information on risk factor operationalization.)

Statistical Analysis

To address the skewed distribution of HOMA-IR values, insulin resistance was operationalized as natural log-transformed HOMA-IR (lnHOMA-IR) in the analyses. Geometric means are presented after back-transforming predicted means obtained in regression analyses described below. Multivariable models regressed continuous measures of lnHOMA-IR, plasma glucose levels, SBP, and DBP (dependent variables) on the continuous summary score for nitrate-reducing bacteria (NO_3TSS) in separate regressions for each outcome. Results were also presented visually in categories of increasing intervals of SD. Because the outcomes of prediabetes mellitus and hypertension were common in our study population, relative risk regression models using a modified Poisson regression with robust error variance were used to calculate the prevalence ratios instead of odds ratios.34 To avoid the possibility of behavioral modification and medications (after a hypertension diagnosis) from masking the associations with bacteria, sensitivity analyses for SBP and DBP outcomes were conducted using only the 187 participants with normotension. Exploratory analyses of the relationship between the 20 individual bacteria taxa and the cardiometabolic outcomes of interest were also conducted.

All multivariable regressions were adjusted for the potential confounders of age, sex, race, and smoking status a priori based on previous studies, with education, body mass index, percentage of probing sites with attachment loss ≥3 mm, and dietary pattern additionally included as they were associated with the exposure and outcomes at an α=0.20 level of significance (Table S3). Additional sensitivity analysis was also conducted in which alcohol use and physical activity were added to the regression model.

Results

Sample Characteristics

The demographic characteristics of the ORIGINS cohort (n=281) are presented in Table S4. The mean age of our study population was 34 years (SD=10 years), and the majority were women (78%), college educated (67%), and never smokers (79%). A total of 42% of participants had none or mild periodontitis as defined per the Centers for Disease Control and Prevention/American Academy of Periodontology guidelines.35 A total of 95% of the a priori–selected sites from which subgingival plaque was sampled had a probing depth ≤3 mm, and the remaining 5% (11 sites) had a probing depth of 4 mm. The prevalence of prediabetes mellitus and hypertension in this population was 18% (n=50) and 33% (n=93), respectively. The characteristics of the participants with normotension were comparable to the whole sample (Table S4).

Prevalence and Relative Abundance of Individual Nitrate-Reducing Bacteria

The mean relative abundances and prevalence of the 20 individual nitrate-reducing bacterial taxa are presented in Figure 1. *Rothia dentocariosa* had the highest mean relative abundance (7.9%) and was detected in all participants, whereas *Propionibacterium acnes* had the lowest relative abundance (0.0002%) and was detected in only 6% of participants. However, it should be noted that at such low

relative abundance (<0.0005%) the reliability of this taxa distribution is poor. Participants had a mean total relative abundance of nitrate-reducing taxa of 20% (SD=16%; range: 0.09%–86%), and many of the nitrate-reducing bacteria species were present in most participants. The mean nitrate-reducing bacterial summary score NO_3TSS was ≈0 (SD=5.42).

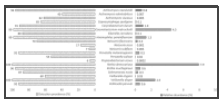

Figure 1

The prevalence (%) and mean relative abundance (%) of the 20 nitrate-reducing taxa measured in subgingival plaque samples among the 281 participants in ORIGINS (Oral Infections, Glucose Intolerance, and Insulin Resistance Study).

Association of Nitrate-Reducing Bacterial Summary Score With Insulin Resistance, Plasma Glucose, and Prediabetes Mellitus

The mean (IQR) HOMA-IR and mean (SD) plasma glucose values in this population were 1.75 (1.45) and 85 mg/dL (7.6 mg/dL), respectively. A higher NO_3TSS was associated with lower insulin resistance. Every 1-SD higher NO_3TSS, was associated with a −0.09 (95% CI, −0.15 to −0.03) lower lnHOMA-IR, controlling for age, sex, race, education, body mass index, smoking status, percentage of probing sites with attachment loss ≥3 mm, and dietary pattern (Table 1). The geometric means of the HOMA-IR values across increasing SD intervals of NO_3TSS were 1.85 (95% CI, 1.55–2.22), 1.89 (95% CI, 1.66–2.16), 1.59 (95% CI, 1.38–1.83), and 1.46 (95% CI, 1.22–1.73) (linear trend *P*=0.003). Mean values of lnHOMA-IR are presented in Figure 2.

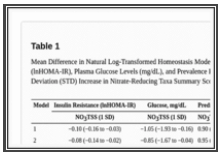

Table 1

Mean Difference in Natural Log-Transformed Homeostasis Model Assessment for Insulin Resistance (lnHOMA-IR), Plasma Glucose Levels (mg/dL), and Prevalence Ratio of Prediabetes for Every 1 Standard Deviation (STD) Increase in Nitrate-Reducing ...

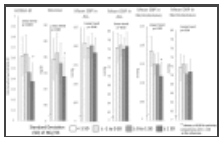

Figure 2

Natural log-transformed homeostatic model assessment of insulin resistance (HOMA-IR) values, plasma glucose, systolic blood pressure (SBP), and diastolic blood pressure (DBP) (95% CI) across increasing SD intervals of nitrate-reducing ...

Inverse associations were also observed between NO_3TSS and baseline plasma glucose (Table 1). In multivariable analyses, every 1-SD higher NO_3TSS was associated with a −1.03 (95% CI, −1.90 to −0.16) lower plasma glucose level (mg/dL). Prediabetes mellitus prevalence tended to decrease as NO_3TSS levels increased (0.79; 95% CI, 0.61–1.03) (Table 1).

Association of Nitrate-Reducing Bacterial Summary Score With BP and Hypertension

The mean (SD) SBP and DBP was 117 mm Hg (12 mm Hg) and 75 mm Hg (10 mm Hg), respectively, in the whole sample; 112 mm Hg (9 mm Hg) and 70 mm Hg (6 mm Hg), respectively, for participants with normotension (n=187) and 128 mm Hg (11 mm Hg) and 84 mm Hg (9 mm Hg), respectively, for participants with hypertension (n=93).

When examining the association between NO_3TSS and BP outcomes in the full sample, NO_3TSS was not significantly associated with SBP or DBP (Table 2). However, in sensitivity analyses including only participants with normotension, the effect estimates were similarly inverse but larger for SBP. A SD higher NO_3TSS was associated with a −1.53 mm Hg (95% CI, −2.82 to −0.24) lower mean SBP (Table 2). Multivariable adjusted mean values of SBP across increasing SD intervals of NO_3TSS were 118 mm Hg (95% CI, 114–122), 115 mm Hg (95% CI, 112–118), 115 mm Hg (95% CI, 112–118), and 112 mm Hg (95% CI, 108–116) (linear trend *P*=0.02) (Figure 2). The association between NO_3TSS and mean DBP was smaller but likewise inverse (−0.60; 95% CI, −1.54 to 0.33) (Table 2). Higher NO_3TSS was not associated with a higher prevalence ratio of hypertension (1.03; 95% CI, 0.89–1.20).

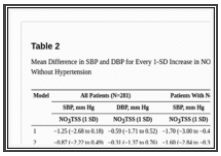

Table 2

Mean Difference in SBP and DBP for Every 1-SD Increase in NO Without Hypertension

Mean Difference in SBP and DBP for Every 1-SD Increase in NO_3TSS in the Full Sample and in Patients Without Hypertension

Sensitivity analyses defining hypertension using old thresholds of SBP ≥140 mm Hg or DBP ≥90 mm Hg[36] found similar results: the prevalence ratio for hypertension was 1.21 (95% CI, 0.91–1.61). Results for SBP and DBP among patients with normotension using these thresholds are presented in Table S5.

Exploratory Analyses for Individual Nitrate-Reducing Bacterial Species

Upon examination of the associations between the individual nitrate-reducing bacterial taxa and cardiometabolic outcomes, a few significant associations were found (Tables S6 and S7). Higher relative abundance of *Neisseria flavescens* and *Haemophilus parainfluenzae* were associated with lower insulin resistance and mean SBP, and lower plasma glucose, SBP, and DBP, respectively. While other taxa such as *Actinomyces naeslundii*, *Actinomyces viscosus*, *Capnocytophaga sputigena*, and *Neisseria sicca* also had some individual associations with lower cardiometabolic outcomes, no bacterial species was consistently associated across the different cardiometabolic outcomes.

Discussion

Among a sample of young diabetes mellitus–free individuals, we found higher relative abundance of nitrate-reducing oral bacteria to be associated with lower insulin resistance and plasma glucose in all participants, and with mean SBP in participants with normotension only, cross-sectionally. These results inform the potential influence of oral bacteria on cardiometabolic outcomes via the enterosalivary nitrate-nitrite-NO pathway of NO generation, and add important knowledge to the nascent literature in this area in a number of meaningful ways.

Unlike previous studies, this study directly examines a broad set of putative nitrate-reducing organisms in relation to cardiometabolic outcomes in a population-based observational setting. Few studies have directly measured the oral microbiota when examining the enterosalivary pathway of NO generation with health outcomes.[20, 21] Of these, none have examined the outcomes of insulin resistance and plasma glucose, and our study utilizes the largest sample size to date. Our findings demonstrate that a meaningful proportion (≈20%) of oral taxa are potentially nitrate-reducing, while also showing substantial between-person variation in the relative abundance of nitrate-reducing bacteria. Moreover, the results suggest that higher levels of nitrate-reducing organisms might confer health benefits across the population distribution of bacterial levels. Thus, if this relationship were causal, interventions to manipulate nitrate-reducing bacterial levels may be a useful treatment modality to improve cardiometabolic health, even in younger, generally healthy populations. The difference of ≈3 to 6 mm Hg in mean SBP observed between the highest and lowest SD intervals of NO_3TSS in our study is comparable to the estimated effects (5.7 mm Hg for SBP and 3.1 mm Hg for DBP) of first-line antihypertensive medications.[37] Importantly, a 4.4-mm Hg reduction in SBP has been estimated to reduce the risk of cardiovascular events by as much as 14%.[37] The smaller differences in insulin resistance and fasting plasma glucose currently observed between SD intervals of nitrate-reducing bacteria have not previously been associated with an increased conversion to overt diabetes mellitus or cardiovascular disease incidence. But future studies with longer follow-up times that allow for the development of greater impairment of glucose regulation may yield greater clinical relevance.

The lack of an association found between NO_3TSS and prediabetes mellitus or hypertension is inconsistent with the findings for insulin resistance, glucose, and BP in our study. It is possible that nitrate-reducing bacteria may be most relevant in the early preclinical stages of disease development, before more advanced pathophysiological alterations (eg, reduced β-cell function or increased arterial stiffness) occur and environmental risk factors lose importance. Furthermore, behavioral changes (eg, improved diet and activity levels) or medical therapies following the diagnosis of hypertension, prediabetes mellitus, or other comorbidities (eg, high cholesterol) could favorably influence both the nitrate-reducing bacteria and cardiometabolic health, masking these associations. This notion is supported by the observation that NO_3TSS was most strongly associated with SBP among individuals with normotension only. Alternatively, these observations may simply be the result of chance, and replication in future studies will be important.

Study Limitations

Some important limitations should be noted. Because of the cross-sectional design of our study, reverse causation is possible. Insulin resistance, plasma glucose, and BP levels could all influence microbial community composition, even in the clinically normal range. High salivary glucose is associated with a shift in the composition of the oral microbiome, although the direct influence on nitrate-reducing bacteria is unknown and studies have mostly considered only glucose levels in the diabetic range.[38]

Measurement error in the assessment of nitrate-reducing bacteria might have diluted the strength of association as the day-to-day stability of the oral microbiome is unclear.39 ORIGINS only measured 20 of the 28 bacterial species previously identified as being associated with nitrate-reduction capacity. Since there are many more oral bacteria with nitrate-reducing capacity, it is also likely that not all relevant nitrate-reducing bacteria have been identified, as only 2 studies have sought to identify the key contributors to oral nitrate-reduction.25, 26 Additionally, strain-level variation within the same species, horizontal gene transfer between bacteria,40 and different rates of nitrate-reduction across taxa25, 26 may all result in the misclassification of the individual's nitrate-reducing capacity. Metagenomics to directly assess the genes (eg, *narG, narL, napC, napB*) encoding for the nitrate-reductase produced by bacteria,26 or metatranscriptomics measuring gene expression, may better capture the nitrate-reducing capacity. Likewise, including a measure of plasma or salivary nitrite together with the bacteria measures can further support the increase in nitrite production through these bacteria and may be useful for mediation analyses. Although the amount of salivary nitrate reduced to nitrite by oral bacteria appears to be substantial and dose-depen dent,19, 21 the percentage that reaches the systemic circulation as plasma nitrite is unclear but appears to be less, and a possible saturation threshold has been suggested that needs further examination.17, 21 Nevertheless, misclassification of the nitrate-reducing bacteria exposure is likely nondifferential by the outcomes, biasing the estimates towards the null.

Nitrate-reducing bacteria are present in various sites of the oral cavity. Our study was only able to examine subgingival bacteria when the tongue is believed to be the main site of bacterial nitrate reduction in the mouth.25 The oral cavity is thought to contain distinct niches of microbiota with varying microbial diversity and composition,41 and it is unknown whether levels of nitrate-reducing bacteria in the subgingival plaque serve as a reasonable proxy for levels on the tongue. The assessment of subgingival microbiota from one periodontal site per participant, as in this study, is also highly likely to have increased measurement error of the full-mouth exposure to nitrate-reducing taxa, which would bias the results towards the null. Future studies that can directly assess nitrate-reducing bacteria on the tongue will be important.

Our results also do not account for pathways involving the gut. As contiguous parts of the digestive tract, the gut, and oral microbiota likely influence one another.42 The gut microbiome is also capable of nitrate reduction, although its contribution to circulating nitrite is likely minor, as the main site of nitrate to nitrite reduction occurs in the mouth, and dietary nitrate is mostly absorbed from the proximal intestine into the circulation, bypassing the nitrate-reducing bacteria residing more distally. However, gut bacteria (eg, *Lactobacilli* sp., *Bifidobacterium*) can directly produce NO, potentially influencing blood flow and mucus generation, and thus the uptake and bioavailability of nitrate and nitrite.10 Gastric pH is also of relevance with high levels of NO formed nonenzymatically in the acidic stomach from swallowed nitrite and acid-reducing medication shown to attenuate the BP-lowering effects of nitrate.43 Furthermore, the gut microbiome produces hydrogen sulfide (H_2S), another physiologically important gaseous signaling molecule, involved in the formation of NO from nitrite in the intestine and systemic tissues.10 Evidence suggests that an interplay of H_2S and NO has cardiovascular effects.44 Thus, the gut microbiome may modify nitrite and NO bioavailability, and more research is needed to fully understand the role of the gut microbiome in the nitrate-nitrite-NO enterosalivary pathway.

Future studies that consider the role of oral nitrite reduction will also be important. Oral bacteria can further reduce salivary nitrite to NO, influencing the amount of bioavailable salivary nitrite swallowed and absorbed into the systemic circulation.25, 26 Thus, the optimal oral bacterial community for NO generation may be one that allows for nitrite accumulation, and the ratio of the nitrate versus nitrite-reducing capacity of the oral microbiome, the exposure of greatest interest.26, 45 Furthermore, a correlation between higher bacterial nitrite-reductase gene abundance and lower resting SBP was recently found, suggesting that orally produced NO may have systemic effects on vasodilation as well.45 However, few other studies have specifically explored the nitrite-reducing capacity of the oral cavity, and to the best of our knowledge the key bacterial species contributing to nitrite reduction in the mouth have yet to be identified.

Finally, although frequent regular mouthwash use has been associated with an increased risk of prediabetes mellitus,46 the ORIGINS questionnaire did not contain information on mouthwash use and we were unable to control for mouthwash use.

Study Strengths

Despite these limitations, several strengths should be noted. ORIGINS collected a robust set of risk factor data allowing for comprehensive control for hypothesized confounders. The use of next-generation sequencing techniques allowed for more precise identification of a larger set of nitrate-reducing bacteria, and the relatively young cohort, free of diabetes mellitus and other clinical cardiovascular diseases, minimizes reverse causality.

Conclusions

This is one of the first studies to directly test the hypothesis of a priori–identified nitrate-reducing oral bacteria affecting cardiometabolic outcomes. Our results support the hypothesis that oral nitrate-reducing bacteria play a beneficial role in BP regulation and insulin resistance. Future longitudinal studies with enhanced assessment of nitrate-reducing bacterial exposure predicting progression of cardiometabolic risk biomarkers and incident clinical disease will improve temporal inference necessary to inform causality and inform the development of future intervention studies that could manipulate oral nitrate-reducing capacity.

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Disclosures

None.

Supporting information

Data S1. Supplemental methods.

Table S1. List of Previously Identified Oral Bacterial Species or Genera With Potential Nitrate-Reducing Capacity

Table S2. Spearman Correlations Between the Mean Relative Abundance for Each of the 20 Nitrate-Reducing Taxa and Summary Scores With (ie, NO_3TSS) or Without Standardization

Table S3. Associations Between Potential Confounders and (**A**) Nitrate-Reducing Bacterial Summary Score (NO_3TSS), Insulin Resistance, (**B**) Fasting Glucose, Blood Pressure, and (**C**) Prediabetes Mellitus and Hypertension

Table S4. Characteristics of the 281 Participants From Oral Infections, Glucose Intolerance, and Insulin Resistance Study (ORIGINS) Used in This Study

Table S5. Mean Difference in Systolic and Diastolic Blood Pressure (mm Hg) for Every 1 Standard Deviation (STD) Increase in Nitrate-Reducing Bacterial Summary Score (NO_3TSS) in Patients Without Hypertension (n=242) According to the 2003 American Heart Association (AHA) Guideline's 140/90 mm Hg Cutoff for Hypertension, and in Patients Who had Hypertension and Were Taking Medication (n=13)

Table S6. Mean Difference in Natural Log-Transformed Homeostasis Model Assessment for Insulin Resistance (lnHOMA-IR) and Plasma Glucose, and Prevalence Ratio for Prediabetes Mellitus, for Every 1-SD Higher *z* Score Arcsin-Square Root–Transformed Relative Abundance for the 20 Individual Nitrate-Reducing Bacteria Taxa

Table S7. Mean Difference in Mean Systolic and Diastolic Blood Pressure, and Prevalence Ratio for Hypertension, for Every 1 Standard Deviation Higher *z*-Score Arcsin-Square Root Transformed Relative Abundance for the 20 Individual Nitrate-Reducing Bacteria Taxa

Figure S1. Spearman correlations of the relative abundance levels for 20 nitrate-reducing subgingival bacterial species (n=281).

Click here for additional data file.(328K, pdf)

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