Published in final edited form as:

J Periodontol. 2014 July ; 85(7): 944–955. doi:10.1902/jop.2013.130518.

Association of plasma 25-hydroxyvitamin D concentrations and pathogenic oral bacteria in postmenopausal women

Michelle W Sahli, MS^{*}, Jean Wactawski-Wende, PhD^{*}, Pavani K Ram, MD^{*}, Michael J. LaMonte, PhD^{*}, Kathleen M. Hovey, MS^{*}, Robert J. Genco, PhD, DDS[†], Christopher A. Andrews, PhD[‡], and Amy E. Millen, PhD^{*}

^{*}Department of Social and Preventive Medicine, School of Public Health and Health Professions, University at Buffalo, The State University of New York, Buffalo, NY

[†]Department of Oral Biology, School of Dental Medicine, University at Buffalo, The State University of New York, Buffalo, NY

[‡]Department of Ophthalmology & Visual Science, Medical School, University of Michigan, Ann Arbor, MI

Abstract

Background—Previous findings of an association between 25-hydroxyvitamin D (25(OH)D) concentrations and periodontal disease, may be partially explained by vitamin D's antimicrobial properties. To our knowledge, no study has investigated the association between 25(OH)D and pathogenic oral bacteria, a putative cause of periodontal disease.

Methods—We examined the association between plasma 25(OH)D concentrations and pathogenic oral bacteria among postmenopausal women in the Buffalo Osteoporosis and Periodontal Disease Study (1997–2000), an ancillary study of the Women's Health Initiative Observational Study. Subgingival plaque samples were assessed using immunofluorescence for the presence of *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Campylobacter rectus*. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for prevalent bacteria by quintile (Q) of 25(OH)D concentrations adjusting for age and body mass index.

Results—Of the 855 participants, 288 (34%) had deficient/inadequate (<50 nmol/L) 25(OH)D concentrations and 497 (58%) had at least one species of pathogenic bacteria. No significant association was found between 25(OH)D and presence of any of these bacteria (adjusted OR for high (Q5) compared to low (Q1) 25(OH)D=0.96; 95% CI: 0.61–1.50, p for trend=0.50). Inverse, although not statistically significant, associations were found between 25(OH)D and more than one species of pathogenic bacteria (adjusted OR for adequate compared to deficient/inadequate 25(OH)D=0.85; 95% CI: 0.60–1.19).

Corresponding Author: Amy E. Millen, Department of Social and Preventive Medicine, School of Public Health and Health Professions, University at Buffalo, The State University of New York, 270 Farber Hall, Buffalo, NY 14214-8001. Telephone: (716) 829-5377, Fax: (716) 829-2979, aemillen@buffalo.edu.

AUTHOR DISCLOSURES AND CONFLICTS OF INTEREST

M.W.Sahli, J. Wactawski-Wende, P.K. Ram, M.J. LaMonte, K.M. Hovey, C.A. Andrews and R.J. Genco have no conflicts of interests or disclosures to report.

Conclusions—No association was observed between pathogenic oral bacteria and 25(OH)D concentrations in postmenopausal women. This may be due to the species of bacteria assessed, small effect size or a true absence of an association.

Keywords

vitamin D; 25-hydroxyvitamin D; periodontal diseases; dental plaque; periodontitis; postmenopause

Introduction

Inflammation of the oral gingiva found in persons with periodontal diseases is thought to be caused by the immune system's response to bacterial infection.^{1, 2} Gingival inflammation (aka. gingivitis), assessed by the degree of gingival bleeding, is the least severe form of periodontal disease, but can also be present in people with more severe periodontal disease. Vitamin D has been hypothesized to protect against gingival bleeding by two potential mechanisms: suppression of inflammation^{2, 3} and/or upregulation of antimicrobial peptide transcription^{4, 5}.

Limited data has been published on the association between vitamin D status and gingivitis. Using data from the Third National Health and Nutrition Examination Survey (NHANES III, 1988–94), Dietrich et al. observed an inverse dose-response association between serum 25-hydroxyvitamin D (25(OH)D) concentrations and gingival bleeding upon probing.⁶ In a cross-sectional study of postmenopausal women, Millen et al. found that plasma 25(OH)D concentrations were inversely associated with the percent of gingival sites that bleed upon assessment.⁷ Because of the established etiological role subgingival bacteria has in periodontal disease, and because 25(OH)D is thought to have antimicrobial properties, it is possible that the observed inverse association between vitamin D status and gingival bleeding was, in part, by way of an association with oral bacteria. We found no published studies to date that have directly tested this hypothesis.

The purpose of this study was to evaluate whether the association between plasma 25(OH)D concentrations and gingival bleeding previously reported^{6, 7} could be explained, at least in part, by vitamin D's postulated antimicrobial properties. To test this hypothesis, we examined whether there was a cross-sectional association between plasma 25(OH)D concentrations and presence of pathogenic oral bacteria among 855 postmenopausal women with both plasma 25(OH)D concentrations and five pathogenic oral bacteria (determined by immunofluorescence) measured from samples collected at a single study visit conducted between 1997 and 2000.

Materials and Methods

Study Design

Our study uses information collected from the Buffalo Osteoporosis and Periodontal Disease Study (OsteoPerio Study), an ancillary study of the Women's Health Initiative (WHI) Observational Study (OS) conducted among women enrolled at the Buffalo, NY center. The WHI was designed to examine common health concerns of postmenopausal women

including cancer, heart disease and osteoporosis, as well as other health outcomes.⁸ The study included both a series of randomized clinical trials and an observational study. The OsteoPerio Study was timed to coincide with participants' year three clinic visit in the WHI OS conducted from 1997 to 2000. The primary purpose of the OsteoPerio study was to examine the association between osteoporosis, oral bone loss and periodontal disease in a well-characterized cohort of postmenopausal women.

Study Population

The Buffalo, NY clinical center of the WHI OS enrolled 2,249 participants. These women subsequently were invited by mail to take part in the OsteoPerio Study around the time of their year three clinic visit in the WHI OS. As previously described by Wactawski-Wende et al.⁹, of those invited to participate in the study, 115 could not be reached, 343 stated that they were not interested in participating, 52 were deceased, 27 canceled their appointments and 12 were deemed temporarily ineligible due to recent oral x-rays or radio luminescent dye tests. Of those screened for eligibility, 338 women did not meet the study criteria because they had less than six teeth (162 women), had been diagnosed with cancer in the previous ten years (106 women), had a serious illness at the time of the year three visit (52 women), had had bilateral hip replacement (16 women) or had been diagnosed with bone disease (2 women)⁹. This left 1,362 women who were eligible for and participated in the study.

Of these 1,362 participants, 16 had inadequate study x-rays and 5 either did not complete or return study questionnaires. These women were excluded from this analytic dataset.⁹ Blood collection was added to the study protocol after the study initiation. As such, stored plasma for assays of 25(OH)D were available for 934 of the 1,341 women enrolled. Of those women with plasma available, 78 were missing measures of oral bacteria and one woman was excluded due to her extreme value for plasma 25(OH)D (530 nmol/L) leaving a sample size of 855 for the current analyses.

Plasma 25(OH)D Concentrations

Morning fasting blood samples were collected from participants during their OsteoPerio Study visit. Plasma from these samples was separated according to standard protocols into 0.5 ml cryogenic straws, frozen and stored at -196°C in liquid nitrogen at the time of collection. Samples were taken from liquid nitrogen and placed into a -80°C freezer where they were later thawed, aliquotted into cryovials, refrozen and shipped on dry ice to the laboratory[§], as previously described.¹⁰ Once received by the laboratory, samples were re-thawed and assayed in batches over a four month period. Assays^{||} were performed to quantify plasma 25(OH)D concentrations. Blinded duplicate quality controlled samples were included in each batch and gave a within-pair coefficient of variation of 4.9%.

Collection and Assessment of Periodontal Bacteria

Prior to conducting any oral probing measures, subgingival plaque samples were taken from 12 index teeth or their substitutes using the paper point technique as previously described by Brennan et al.¹¹ The paper points containing the plaque were placed directly in Ringer's solution as they were removed from the mouth. These were sent to the laboratory for

processing where one half of the solution was frozen. The other half was fixed, using 10% neutral buffered formalin, and remained at room temperature until used for the bacterial analysis. For bacterial analysis, samples of this plaque suspension were vortexed and then heat fixed to a slide. The resulting smears were then reacted with species-specific antisera or monoclonal antibodies, washed and incubated with fluorescein-coagulated immunoglobulin G and washed again.¹¹ The stained smears were examined with a specially equipped microscope and fluorescence graded as 1+ through 4+. For the purpose of this study any fluorescence graded as 3+ or 4+ was considered serologically positive. Plaque samples were tested for presence of eight species of bacteria.¹² Five of these bacterial species were chosen because Socransky et al. had classified them as “red complex” (*Porphyromonas gingivalis* and *Tannerella forsythensis*) or “orange complex” (*Fusobacterium nucleatum*, *Prevotella intermedia* and *Campylobacter rectus*) signifying that they were thought to be periodontal pathogens.^{11, 12} “Red complex” bacteria are found in the gingiva of people with the most severe periodontal disease and “orange complex” bacteria are found in those with less severe periodontal disease.^{13, 14} Both *Capnocytophaga* sp and *Streptococcus saburreum* were collected because they were not thought to be associated with periodontal disease. And it was unknown whether or not *Eubacterium saburreum* had any association with periodontal disease.¹²

Oral Health Exam

After collection of subgingival plaque samples, each participant in the OsteoPerio Study was given a clinical oral exam. This comprehensive exam included radiographs for assessment of alveolar crestal height, and probing measure including periodontal probing depth (PPD) and clinical attachment level (as previously described by Brennan et al.¹¹). Briefly, PPD was assessed for six sites of each tooth present, when possible, using a constant-force electronic periodontal probing system[¶] which measures the distance from the gingival margin to the bottom of the periodontal pocket.¹¹ Whole-mouth mean PPD was determined by averaging all PPD measures across all sites in a participant’s mouth.

Questionnaire Data and Physical Measurements

Data used in this study was collected from questionnaires administered during the participants’ WHI OS baseline and third visits. These questionnaires, along with questions specific to the OsteoPerio study, inquired about demographics, lifestyle and oral health habits, as well as systemic and oral health history. OsteoPerio participants were also asked to bring in all medications and supplements used in the last 30 days and the dose, frequency and duration of medications and supplements were recorded. Participants’ height (m) and weight (kg) were assessed with a wall-fixed stadiometer and a calibrated balance beam, respectively. From these data, body mass index (BMI)(kg/m²) was calculated.

Statistical Methods

If *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Fusobacterium nucleatum*, *Prevotella intermedia* or *Campylobacter rectus* were detected, the participant was considered to have pathogenic oral bacteria present which was the primary outcome in our analysis. For our primary analyses, quintiles of 25(OH)D concentrations were created with quintile 1 having

the lowest 25(OH)D concentrations. We examined the distribution of participant characteristics and potential risk factors in the study sample, both according to quintiles of the exposure, plasma 25(OH)D, and presence of any of these pathogenic oral bacteria. We investigated several individual characteristics and risk factors that have been shown to be associated with gingival bleeding or periodontal disease in previously published literature as follows: age, race, BMI, frequency of tooth brushing, frequency of flossing, frequency of dental visits, days since last dental cleaning, whole-mouth mean PPD, self-reported history of diabetes, smoking, alcohol consumption, antibiotic use in the 30 days prior to the study visit, current use of hormone therapy drugs and self-reported total recreational physical activity. Analysis of the distribution of these characteristics and risk factors within the study population by exposure category or outcome status was performed using chi-square tests for categorical variables and t-tests or ANOVAs for continuous variables. Two sided p-values 0.05 were considered statistically significant.

Next, we evaluated the association between 25(OH)D concentrations and presence of pathogenic oral bacteria using logistic regression. Unadjusted odds ratios (ORs) and 95% confidence intervals (CIs) for presence of oral pathogenic bacteria (yes/no) were estimated for participants in quintiles 2, 3, 4 and 5 compared to quintile 1 (reference group) of 25(OH)D. We then constructed an adjusted model by forward selection where we considered potential confounders to be variables univariately associated with both the exposure and outcome at p -value 0.20. Each potential confounder was added to the crude model separately. The variable that influenced the OR for the association between 25(OH)D and pathogenic bacteria to the greatest extent was included in the logistic regression model provided it changed the OR by 10%. Next, additional potential confounders were added sequentially to determine if they further influenced the OR by 10% or more. This stepwise process continued until addition of no other potential confounders influenced the OR 10%.

Opinions vary concerning what indicates an adequate concentration of 25(OH)D. The Institute of Medicine uses a cutoff point of 50nmol/L as adequate with respect to bone related outcomes.¹⁵ However, ideal concentrations of 25(OH)D may depend on the condition being studied.¹⁶ For this reason, we conducted additional analyses using clinically defined cut points: deficient (<30nmol/L), inadequate (30 to <50nmol/L), adequate (50 to 75 nmol/L) and adequate (>75nmol/L).¹⁵ Whether or not 25(OH)D concentrations above 75nmol/L impart any additional benefit on health outcomes has not been determined and therefore was investigated in these analyses.¹⁵

Additionally, in a restricted analysis, we removed participants who did not have the full possible twelve teeth sampled for plaque to examine whether potential outcome misclassification from limited teeth sampling may have influenced our results. We also conducted additional exploratory analyses estimating the odds ratios of having more than one pathogenic, all orange complex, or all red complex bacterial species among participants with adequate (≥ 50 nmol/L) compared to inadequate (<50 nmol/L) 25(OH)D concentrations.¹⁷

We hypothesized that women with low levels of oral hygiene may benefit more from high concentrations of vitamin D than those with high levels of oral hygiene, and therefore

conducted an exploratory analysis assessing the interaction between plasma 25(OH)D concentrations and oral hygiene level. We chose frequency of flossing to represent level of oral hygiene because it was the dental hygiene variable most strongly associated with oral bacteria. We also explored interactions between 25(OH)D concentrations and whole-mouth mean PPD, smoking status, current use of antibiotics, current hormone therapy use and self-reported history of diabetes. A p-value for interaction <0.10 was considered statistically significant.

Results

Mean 25(OH)D concentrations within quintile exposure groups ranged from 29.4 ± 7.8 (mean $\pm$ SD) nmol/L in quintile one (Q1) to 95.0 ± 36.5 nmol/L in quintile five (Q5) (Table 1). Women in Q5 were, on average, slightly younger, had more teeth, and a lesser proportion of teeth that bleed upon assessment than women in Q1. Oral hygiene practices were significantly different between women according to quintiles of 25(OH)D. A greater proportion of participants in Q5 than Q1 reported more frequent tooth brushing, flossing and dental visits. A greater proportion of participants in Q5 than Q1 had lower BMIs, used hormone therapy, did not self-report a history of diagnosed diabetes, and, on average, reported greater levels of total recreational physical activity.

There were 496 (58.1%) women in whom we detected the presence of at least one the five species of pathogenic bacteria (Table 2). Although 25(OH)D concentrations were, on average, lower in those with as compared to without any pathogenic bacteria (59.3 vs. 61.2 nmol/L), this difference was of a small magnitude and not statistically significant. Women with pathogenic bacteria had, on average, a greater proportion of teeth that bleed upon assessment and a higher whole-mouth mean PPD as compared to women with no pathogenic bacteria. A greater proportion of women with pathogenic bacteria flossed infrequently, had larger BMIs, were never smokers, and did not use hormone therapy compared to those without pathogenic bacteria.

In the crude analysis, high compared to low 25(OH)D concentrations were inversely, but not significantly, associated with presence of pathogenic oral bacteria (OR for Q5 compared to Q1: 0.78; 95% CI: 0.51, 1.20, p for trend=0.18) (Table 3). Only BMI changed the crude association between 25(OH)D and pathogenic bacteria by more than 10%. We also added age to the adjusted model because it has been shown to be a strong predictor of periodontal disease in the literature.¹⁸ After adjustment for age and BMI, the association was attenuated (OR for Q5 compared to Q1=0.96; 95% CI: 0.61–1.50, p for trend=0.50). Results were similar when using clinically defined cut points of 25(OH)D and when the analysis was restricted to include only women who had a complete set of twelve teeth sampled (adjusted OR for Q5 compared to Q1: 0.98; 95% CI: 0.54–1.50, p for trend=0.50). Since the cut point of 75 nmol/L of 25(OH)D may not have been high enough to show an association between 25(OH)D and oral bacteria we also conducted a sensitivity analysis using a higher cut point of 100 nmol/L and found an inverse association (adjusted OR = 0.56 comparing women with 100nmol/L of 25(OH)D to those with <30 nmol/L) although this was not statistically significant (95% CI = 0.28– 1.11).

We further examined associations between 25(OH)D and women with more than one pathogenic bacteria, all orange complex bacteria or all red complex bacteria. Although the results were not statistically significant, we found a stronger, inverse association between 25(OH)D concentrations and presence of more than one pathogenic bacteria (adjusted OR for adequate compared to deficient/inadequate 25(OH)D concentrations: 0.85; 95% CI: 0.60–1.19), all orange complex bacteria (adjusted OR: 0.68; 95% CI: 0.37–1.32) and all red complex bacteria (adjusted OR: 0.80; 95% CI: 0.48–1.33) than in our primary analysis.

In exploratory analyses we examined whether associations between 25(OH)D concentrations and pathogenic oral bacteria were modified by frequency of flossing, whole-mouth mean PPD, smoking status, recent use of antibiotics, hormone therapy use and self-reported history of diabetes (Table 4). We observed a slightly stronger association in women who flossed once a week (adjusted OR: 0.61; 95% CI: 0.35–1.09) compared to those who flossed >once a week (adjusted OR: 1.11; 95% CI: 0.65–1.89) or everyday (adjusted OR: 1.13; 95% CI: 0.69–1.85). However, the p-for interaction term was not significant (p-for interaction = 0.22). We did observe a statistically significant interaction by smoking status. OR and 95% CI comparing ≥ 50 with <50 nmol/L 25(OH)D was 0.25 (0.02 – 3.34) in women who reported being current smokers and 1.00 (0.74 – 1.35) in women who reported being non-smokers at the time of the study (p for interaction = 0.03). However the number of current smokers in this study cohort was very small, limiting interpretation of the findings.

DISCUSSION

We examined the cross-sectional association between plasma 25(OH)D concentrations and pathogenic oral bacteria in postmenopausal women participating in the OsteoPerio Study. To our knowledge, the present study was the first to examine the relationship between vitamin D status and pathogenic oral bacteria. The odds of presence of at least one species of pathogenic oral bacteria in women did not differ by quintile of 25(OH)D concentrations and these results did not vary greatly when clinical cut points for 25(OH)D were used. However, inverse, although not statistically significant, associations were found between 25(OH)D concentrations and pathogenic oral bacteria when analyses were limited to women with more than one pathogenic, all orange complex, or all red complex bacteria species. This may suggest that vitamin D status is associated with more severe oral bacteria composition profiles, having multiple species of pathogenic oral bacteria as opposed to at least one.

Although we observed no association between vitamin D status and any pathogenic oral bacteria in our study, there was a strong biological rationale to believe that one might exist. Previous research in cross-sectional studies^{6, 7} have shown inverse associations between 25(OH)D concentrations and gingival bleeding, a marker of gingival inflammation. Because vitamin D has been shown to have both antimicrobial and anti-inflammatory properties,^{3–5, 18} it has been hypothesized that vitamin D would decrease gingival bleeding or gingivitis through reduction of oral bacteria, inflammation or a combination of the two.

The antimicrobial properties of vitamin D have been characterized. In the presence of pathogens, toll like receptors (TLR) on monocytes and macrophages, activate genes in the

vitamin D pathways including *CYP27B1* (which encodes for 1, 25-alpha hydroxylase) and the vitamin D receptor (*VDR*).⁴ Increased production of 1, 25-alpha hydroxylase has been shown to increase production of cathelicidin, an antimicrobial peptide.^{4, 5} Data supporting vitamin D's proposed antimicrobial effect has been found in both animal¹⁹ and human studies^{20, 21}.

We also investigated several factors that could potentially modify the association between 25(OH)D concentrations and presence of pathogenic oral bacteria. We hypothesized that the association between vitamin D and oral bacteria would be greater in those with poor, as compared to those with better dental hygiene practices as those with poor dental hygiene would have a greater load of oral bacteria. We did observe a more inverse, but not statistically significant association in women with less frequent flossing, but the p for interaction was 0.22. It is probable that a wider variation in dental hygiene practices, or a larger sample size, may have demonstrated an association between vitamin D and oral bacteria in some strata of the population. We did observe a statistically significant interaction between vitamin D status and current smoking status with an inverse association between vitamin D status and oral bacteria in smokers. While on one hand this observation suggests that smokers may be a population subgroup in which sufficient levels of plasma vitamin D may confer a protective effect, the small relative frequency of smokers and the wide confidence interval for the stratum specific OR require that this observation be interpreted and generalized cautiously.

Our findings may indicate an actual absence of an association between 25(OH)D concentrations and pathogenic bacteria, however, this study was a secondary analysis of data from the OsteoPerio Study which was not initially designed to examine the relationship between vitamin D and bacteria. It is possible that 25(OH)D concentrations have an effect on pathogenic oral bacteria that is too small for this study to detect. It is also conceivable that higher concentrations of 25(OH)D are associated with less pathogenic bacteria. We conducted a sensitivity analysis that found an inverse yet not statistically significant association between 25(OH)D concentrations and prevalence of pathogenic bacteria, however it is difficult to draw conclusions from this analysis because only 37 out of 855 participants had 25(OH)D concentrations above 100 nmol/L.

It is also hard to know whether adjustment for BMI led to overadjustment of our association because anthropometric measures of body fatness have been shown to be a strong predictor of vitamin D status in this cohort of postmenopausal women.²² It is also possible that our assay was not able to detect bacteria in those participants who had less than the full twelve paper point subgingival samples taken. However restricting our analyses to include only participants with a complete set of twelve paper points sampled did not alter our results.

Additionally, some (~24%) of the participants in the NY clinical center of the WHI OS, were not available for screening into the OsteoPerio study and others (~15%) did not meet eligibility criteria due to having less than six teeth or certain health conditions. We do not have any information on the 25(OH)D or oral bacteria status of these participants. Our results may have been different if we had been able to include these women in our study.

Since we did not measure quantities of each bacteria species present, we were unable to investigate whether 25(OH)D concentrations were associated with quantity of pathogenic oral bacteria. It is possible that 25(OH)D has an antimicrobial effect sufficient enough to lower the quantity of bacteria present to less than the infectious dose but still above our assay's limit of detection. If this is the case, we might see an inverse association of 25(OH)D concentrations and gingival bleeding, as described by Millen et al.⁷, but not see an inverse association with the presence and absence of oral bacteria. This is supported by our exploratory analyses which suggest that vitamin D status may be more strongly associated with oral bacterial load (e.g., presence of more than one pathogenic bacteria).

Although we measured species of bacteria that have been shown to be associated with periodontal disease¹⁴, the oral cavity can contain over 500 species of bacteria, both pathogenic and nonpathogenic.²³ We conducted assays to detect just five pathogenic species of bacteria. Although the antimicrobial effect of vitamin D on bacteria may not be species specific, it is possible that some bacteria may be more prone to its effects. DNA sequencing of bacteria and other pathogens from the oral cavity would provide a more comprehensive description of the species of bacteria present in study participants. The association between vitamin D status and oral bacteria should be further investigated within these studies.

These data may not be generalizable to other populations as our participants were predominately Caucasian, well-educated, in good health and reported high levels of oral hygiene practices. Our study was also cross-sectional in nature, measuring both vitamin D concentrations and presence of oral bacteria at the same point in time. It is possible that past levels, rather than current levels of 25(OH)D, are related to oral pathogenic bacteria. This seems unlikely, however, since gingivitis, for which bacteria is a necessary cause, is an acute disease often appearing just days after cessation of oral hygiene.²⁴ Even so, previous analysis of these data show that women's plasma 25(OH)D concentrations remain relatively stable over an approximate 5 year period.¹⁰

Despite some noted limitations, the present study has a number of strengths. The OsteoPerio Study was conducted within a large well-established cohort and collected data on a large number of covariates which we were able to investigate as potential confounders. This study is the first to investigate the association between vitamin D status and laboratory identified oral pathogenic bacteria. Intriguing exploratory analyses suggest that vitamin D status may be associated with oral bacteria composition profiles (e.g. those with more than one pathogenic species of bacteria) or within certain subgroups of the population (those with poor oral hygiene and smokers). We were not confined to using less precise measures of vitamin D (e.g., dietary intake), but had measures of 25(OH)D concentrations reflecting intake from all oral sources of vitamin D and sunlight exposure over an approximate three week time period prior to the blood draw.²⁵ Further, we collected both blood and gingival plaque samples at the same time. This was a strength as we hypothesized that recent plasma 25(OH)D concentrations were more relevant to presence of pathogenic oral bacteria than past concentrations.

In conclusion, we observed a lack of association between five species of pathogenic oral bacteria and 25(OH)D concentrations in postmenopausal women in the OsteoPerio Study.

These findings may be due to the species of bacteria assessed, small effect size or a true absence of an association. Further studies are needed to specifically investigate this association using techniques that can better identify and quantify oral bacteria.

Acknowledgments

Sources of Support

This research is supported by NIH grants 1R21DE020918 (awarded to AE Millen) and 1R01DE13505 (awarded to J Wactawski-Wende) from the National Institute of Dental and Craniofacial Research (NIDCR) and a grant awarded to J Wactawski-Wende from the Department of Defense (DAMD179616319).

The WHI program is funded by the National Heart, Lung, and Blood Institute, National Institutes of Health, U.S. Department of Health and Human Services through contracts HHSN268201100046C, HHSN268201100001C, HHSN268201100002C, HHSN268201100003C, HHSN268201100004C, and HHSN271201100004C. We also thank the women who donated their time to participate in the OsteoPerio Study.

A.E. Millen is currently a Co-Investigator on a vitamin D grant funded by the Mushroom Council (#10008).

Appendix

Program Office: (National Heart, Lung, and Blood Institute, Bethesda, Maryland) Jacques Rossouw, Shari Ludlam, Dale Burwen, Joan McGowan, Leslie Ford, and Nancy Geller

Clinical Coordinating Center: (Fred Hutchinson Cancer Research Center, Seattle, WA) Garnet Anderson, Ross Prentice, Andrea LaCroix, and Charles Kooperberg

Investigators and Academic Centers: (Brigham and Women's Hospital, Harvard Medical School, Boston, MA) JoAnn E. Manson; (MedStar Health Research Institute/Howard University, Washington, DC) Barbara V. Howard; (**Stanford Prevention Research Center, Stanford, CA**) Marcia L. Stefanick; (The Ohio State University, Columbus, OH) Rebecca Jackson; (University of Arizona, Tucson/Phoenix, AZ) Cynthia A. Thomson; (University at Buffalo, Buffalo, NY) Jean Wactawski-Wende; (University of Florida, Gainesville/Jacksonville, FL) Marian Limacher; (University of Iowa, Iowa City/Davenport, IA) Robert Wallace; (University of Pittsburgh, Pittsburgh, PA) Lewis Kuller; (Wake Forest University School of Medicine, Winston-Salem, NC) Sally Shumaker

Women's Health Initiative Memory Study: (Wake Forest University School of Medicine, Winston-Salem, NC) Sally Shumaker

REFERENCES

1. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet*. 2005; 366:1809–1820. [PubMed: 16298220]
2. National Center for Biotechnology Information. [Accessed: September 1, 2011] A.D.A.M. Medical Encyclopedia [Internet]. 2011. Available at: <http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001196/>.
3. Guillot X, Semerano L, Saidenberg-Kermanac'h N, Falgarone G, Boissier MC. Vitamin D and inflammation. *Joint Bone Spine*. 2010; 77:552–557. [PubMed: 21067953]
4. Liu PT, Stenger S, Li HY, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*. 2006; 311:1770–1773. [PubMed: 16497887]

5. Ganz T. Defensins: Antimicrobial peptides of innate immunity. *Nat Rev Immunol.* 2003; 3:710–720. [PubMed: 12949495]
6. Dietrich T, Nunn M, Dawson-Hughes B, Bischoff-Ferrari HA. Association between serum concentrations of 25-hydroxyvitamin D and gingival inflammation. *Am J Clin Nutr.* 2005; 82:575–580. [PubMed: 16155270]
7. Millen AEHK, Lamonte MJ, Swanson M, et al. Plasma 25-hydroxyvitamin D concentrations and periodontal disease in postmenopausal women. *J Periodontol.* 2013; 84:1243–1256. [PubMed: 23259413]
8. Anderson G, Cummings S, Freedman LS, et al. Design of the Women's Health Initiative Clinical Trial and Observational Study. *Controlled Clin Trials.* 1998; 19:61–109. [PubMed: 9492970]
9. Wactawski-Wende J, Hausmann E, Hovey K, Trevisan M, Grossi S, Genco RJ. The association between osteoporosis and alveolar crestal height in postmenopausal women. *J Periodontol.* 2005; 76:2116–2124. [PubMed: 16277584]
10. Meng JE, Hovey KM, Wactawski-Wende J, et al. Intraindividual variation in plasma 25-hydroxyvitamin D measures 5 years apart among postmenopausal women. *Cancer Epidemiol Biomarkers Prev.* 2012; 21:916–924. [PubMed: 22523182]
11. Brennan RM, Genco RJ, Hovey KM, Trevisan M, Wactawski-Wende J. Clinical attachment loss, systemic bone density, and subgingival calculus in postmenopausal women. *J Periodontol.* 2007; 78:2104–2111. [PubMed: 17970676]
12. Brennan RM, Genco RJ, Wilding GE, Hovey KM, Trevisan M, Wactawski-Wende J. Bacterial species in subgingival plaque and oral bone loss in postmenopausal women. *J Periodontol.* 2007; 78:1051–1061. [PubMed: 17539719]
13. Socransky SS, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000.* 2005; 38:135–187. [PubMed: 15853940]
14. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL. Microbial complexes in subgingival plaque. *J Clin Periodontol.* 1998; 25:134–144. [PubMed: 9495612]
15. IOM (Institute of Medicine). Dietary Reference Intakes for calcium and vitamin D. Washington DC: The National Academy Press; 2011. Summary; p. 1-14.
16. Bischoff-Ferrari HA, Giovannucci E, Willett WC, Dietrich T, Dawson-Hughes B. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. *Am J Clin Nutr.* 2006; 84:18–28. [PubMed: 16825677]
17. Albandar JM. Global risk factors and risk indicators for periodontal diseases. *Periodontol 2000.* 2002; 29:177–206. [PubMed: 12102708]
18. Ritchie CS, Kinane DF. Nutrition, inflammation, and periodontal disease. *Nutrition.* 2003; 19:475–476. [PubMed: 12714106]
19. Lagishetty V, Misharin AV, Liu NQ, et al. Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. *Endocrinology.* 2010; 151:2423–2432. [PubMed: 20392825]
20. Bodnar LM, Krohn MA, Simhan HN. Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy. *J Nutr.* 2009; 139:1157–1161. [PubMed: 19357214]
21. Urashima M, Segawa T, Okazaki M, Kurihara M, Wada Y, Ida H. Randomized trial of vitamin D supplementation to prevent seasonal influenza A in schoolchildren. *Am J Clin Nutr.* 2010; 91:1255–1260. [PubMed: 20219962]
22. Millen AE, Wactawski-Wende J, Pettinger M, et al. Predictors of serum 25-hydroxyvitamin D concentrations among postmenopausal women: the Women's Health Initiative Calcium plus Vitamin D Clinical Trial. *Am J Clin Nutr.* 2010; 91:1324–1335. [PubMed: 20219959]
23. Marsh, PD.; Martin; Micheal, V. Oral Microbiology. Vol. 222. Edinburgh, London, New York, Oxford, Philadelphia, St. Louis, Sydney, Toronto: Churchill Livingstone Elsevier; 2009.
24. Loe H, Theilade E, Jensen SB. Experimental gingivitis in man. *J Periodontol.* 1965; 36:177–187. [PubMed: 14296927]
25. Zerwekh JE. Blood biomarkers of vitamin D status. *Am J Clin Nutr.* 2008; 87:1087S–1091S. [PubMed: 18400739]

Study summary: In our cross-sectional study in postmenopausal women, vitamin D status was not associated with presence of pathogenic oral bacteria.

Baseline (1997–2000) characteristics of women in the Osteoporosis and Periodontal Disease Study with baseline measurements of both plasma 25(OH)D and pathogenic oral bacteria according to quintiles of plasma 25(OH)D concentrations (N=855)

Table 1

Characteristic	Quintile 1 n=172	Quintile 2 n=170	Quintile 3 n=173	Quintile 4 n=171	Quintile 5 n=169	p-value*
Plasma 25(OH)D						
Plasma 25(OH)D, nmol/L (quintile range)	5.9 – 40.3	40.4 – 53.9	54.0 – 64.1	64.2 – 77.7	77.9 – 154.4	
Plasma 25(OH)D, nmol/L (mean ± SD)	29.4 ± 7.8	47.2 ± 4.1	59.4 ± 3.0	69.9 ± 4.0	95.0 ± 36.5	<0.001
Demographic Characteristics						
Age (mean ± SD)	66.6 ± 7.2	67.6 ± 6.8	67.3 ± 7.1	66.2 ± 7.1	64.8 ± 7.1	<0.01
Race, n (%)						0.24
White	164 (19.2)	166 (19.5)	168 (19.7)	168 (19.7)	167 (19.6)	
Other	8 (0.9)	4 (0.5)	3 (0.4)	2 (0.2)	3 (0.4)	
Education, n (%)						0.48
High school	38 (22.4)	33 (19.4)	39 (22.7)	33 (19.5)	28 (17.1)	
College	84 (49.4)	69 (40.6)	74 (43.0)	81 (47.9)	79 (48.2)	
Post College	48 (28.2)	68 (40.0)	59 (34.3)	55 (32.5)	57 (34.8)	
Measure of Oral Health						
Number of teeth present (mean ± SD)	22.1 ± 6.3	23.8 ± 5.1	23.6 ± 5.1	23.8 ± 5.2	24.1 ± 3.6	<0.003
Proportion of teeth that bled upon assessment (mean ± SD)	0.42 ± 0.3	0.42 ± 0.3	0.34 ± 0.2	0.31 ± 0.2	0.36 ± 0.2	<0.0001
Whole-mouth mean PPD (mean ± SD)	2.2 ± 0.38	2.2 ± 0.38	2.2 ± 0.4	2.2 ± 0.4	2.2 ± 0.4	0.80
Oral Hygiene Practice						
Frequency of tooth brushing, n (%)						0.009
Once a day	49 (28.5)	43 (25.3)	36 (20.8)	26 (15.2)	42 (24.9)	
Twice a day	85 (49.4)	98 (57.7)	94 (54.3)	101 (59.1)	85 (50.3)	
> Twice a day	33 (19.2)	29 (17.1)	42 (24.3)	44 (25.7)	41 (24.3)	
Frequency of flossing, n (%)						0.045
Not every week	38 (22.1)	34 (20.0)	29 (16.8)	23 (13.5)	36 (21.4)	
Once a week	12 (7.0)	17 (10.0)	16 (9.3)	23 (13.5)	17 (10.1)	
> Once a week	62 (36.1)	45 (26.5)	60 (34.7)	42 (24.6)	39 (23.2)	
Everyday	60 (34.9)	74 (43.5)	68 (39.3)	83 (48.5)	76 (45.2)	
Frequency of dental visits, n (%)						0.041

Characteristic	Quintile 1 n=172	Quintile 2 n=170	Quintile 3 n=173	Quintile 4 n=171	Quintile 5 n=169	p-value*
<i>Never/ only with a problem</i>	27 (15.7)	12 (7.1)	17 (9.8)	10 (5.9)	11 (6.5)	
<i>Once a year</i>	24 (14.0)	27 (15.9)	14(8.1)	28 (16.4)	23 (13.6)	
<i>> Once a year</i>	121(70.4)	131 (77.1)	142 (82.1)	133 (77.8)	135 (79.9)	
Other Health and Lifestyle Characteristics						
Body Mass Index, kg/m ² , n (%)						
<25	48 (27.9)	65 (38.0)	67 (39.0)	92 (53.8)	104 (61.8)	<0.001
25 – 30	60 (34.9)	63 (36.8)	72 (41.9)	55 (32.2)	53 (31.2)	
> 30	64 (37.2)	43 (25.2)	33 (19.2)	24 (14.0)	13 (17.7)	
Current smoking status, n (%)						0.09
<i>Never</i>	93 (54.1)	94 (55.3)	92 (53.2)	88 (51.5)	89 (52.7)	
<i>Former</i>	67 (39.0)	72 (42.4)	79 (45.7)	80 (46.8)	75 (44.4)	
<i>Current</i>	12 (7.0)	4 (2.4)	2 (1.2)	3 (1.8)	5 (3.0)	
Alcoholic beverage consumption [‡] , ounces/day (mean ± SD)	0.35 ± 0.7	0.42 ± 0.7	0.47 ± 0.7	0.46 ± 0.6	0.55 ± 0.8	0.10
Recent antibiotic use, n (%)	10 (5.8)	15 (8.8)	16 (9.3)	11 (6.4)	18 (10.7)	0.45
Hormone therapy use, n (%)	67 (39.2)	80 (47.3)	84 (48.8)	88 (52.4)	98 (58.33)	0.009
Self-reported diagnosis of diabetes, n (%)	11 (6.4)	15 (8.8)	6 (3.5)	5 (2.9)	2 (1.2)	<0.006
Total recreational physical activity, MET· hours [‡] /week (mean ± SD)	10.5 ± 13.1	12.4 ± 14.5	14.1 ± 13.3	16.6 ± 13.7	16.7 ± 15.4	<0.0001

* P-value from ANOVAs for continuous variables and χ^2 tests for categorical variables; participants missing information on a variable were not included in ANOVA nor χ^2 calculation for that variable

[‡] Average ounces of ethanol from wine, beer and liquor per day in the past year

[‡] Metabolic equivalents (MET)-hour = duration of activity (hours) × 3.5 ml of O₂ /kg /min

Table 2

Baseline (1997–2000) characteristics of women in the Osteoporosis and Periodontal Disease Study with baseline measurements of plasma 25(OH)D concentrations and pathogenic oral bacteria according to presence of pathogenic bacteria (N=855)

Characteristic	Pathogenic Oral Bacteria		p-value [†]
	None* n=359 (41.9%)	At least one* n=496 (58.1%)	
Plasma 25(OH)D, nmol/L (mean ± SD)	61.2 ± 23.7	59.3 ± 30.3	0.30
Demographic Characteristics			
Age (mean ± SD)	66.1 ± 7.2	66.8 ± 7.0	0.20
Race, n (%)			0.26
<i>White</i>	352 (98.1)	480 (96.8)	
<i>Other</i>	7 (2.0)	16 (3.2)	
Education			0.67
<i>High School</i>	70 (19.7)	101 (20.7)	
<i>College</i>	159 (44.7)	228 (46.6)	
<i>Post College</i>	127 (35.7)	160 (32.7)	
Measures of Oral Health			
Number of teeth present (mean ± SD)	23.2 ± 5.7	23.6 ± 4.8	0.24
Proportion of teeth that bled upon assessment (mean ± SD)	0.31 ± 0.2	0.41 ± 0.2	<0.0001
Whole mouth mean PPD (mean ± SD)	2.1 ± 0.4	2.3 ± 0.4	<0.0001
Oral Hygiene Practices			
Frequency of tooth brushing, n (%)			0.22
<i>once a day</i>	76 (21.2)	127 (25.6)	
<i>Twice a day</i>	196 (54.6)	267 (53.8)	
<i>> Twice a day</i>	87 (24.2)	102 (20.6)	
Frequency of flossing, n (%)			0.037
<i>Not every week</i>	52 (14.5)	108 (21.8)	
<i>Once a week</i>	39 (10.9)	(9.1)	
<i>> Once a week</i>	103 (28.7)	145 (29.3)	
<i>Everyday</i>	165 (46.0)	196 (39.6)	
Frequency of dental visits, n (%)			0.14
<i>Never or only with a problem</i>	27 (7.5)	50 (10.1)	
<i>Once a year</i>	42 (11.7)	74 (14.9)	
<i>< Once a year</i>	290 (80.8)	372 (75.0)	
Other Health and Lifestyle Characteristics			
Body Mass Index, kg/m ² , n (%)			<0.01
<25	182 (51.0)	193 (38.9)	
25 – 30	112 (31.4)	190 (38.3)	
> 30	63 (17.7)	113 (22.8)	
Current smoking status n (%)			<0.008

Characteristic	Pathogenic Oral Bacteria		p-value [†]
	None* n=359 (41.9%)	At least one* n=496 (58.1%)	
<i>Never</i>	247 (49.8)	209 (58.2)	
<i>Former</i>	228 (46.0)	145 (40.4)	
<i>Current</i>	21 (4.2)	5 (1.4)	
Alcoholic beverage consumption [‡] , ounces/day (mean ± SD)	0.46 ± 0.7	0.44 ± 0.7	0.58
Recent antibiotic use, n (% yes)	32 (8.9)	38 (7.7)	0.51
Hormone therapy use, n (% yes)	203 (57.0)	214 (43.5)	<0.001
Self-reported diagnosis of diabetes, n (% yes)	17 (4.7)	22 (4.4)	0.84
Total recreational physical activity, MET- hours [§] /week (mean ± SD)	14.5 ± 13.7	13.7 ± 14.5	0.39

* of the pathogenic bacteria measured

[†] P-value from ANOVAs for continuous variables and χ^2 tests for categorical variables; participants missing information on a variable were not included in ANOVA nor χ^2 calculation for that variable

[‡] Average ounces of ethanol from wine, beer and liquor per day in the past year

[§] Metabolic equivalents (MET)-hour = duration of activity (hours) × 3.5 ml of O₂ /kg /min

Table 3

Crude and adjusted odds ratios (OR) and 95% confidence intervals (95% CIs) for prevalent pathogenic oral bacteria among women in quintiles 2–5 compared to 1 for plasma 25(OH)D among women in the Osteoporosis and Periodontal Disease Study (1997–2000) (N=855)

Association	# with outcome/ total #	Crude		Adjusted*	
		OR	95% CI	OR	95% CI
Any Pathogenic Bacteria (n=855)					
Quintile of plasma 25(OH)D, nmol/L (quintile range)					
Quintile 1 (5.9 – 40.3)	104/172	1.00	referent	1.00	referent
Quintile 2 (40.4 – 53.9)	107/170	1.11	(0.72 – 1.72)	1.16	(0.75 – 1.81)
Quintile 3 (54.0 – 64.1)	92/173	0.74	(0.48 – 1.14)	0.78	(0.51 – 1.21)
Quintile 4 (64.2 – 77.7)	101/171	0.94	(0.61 – 1.45)	1.09	(0.70 – 1.70)
Quintile 5 (77.9 – 154.4)	92/169	0.78	(0.51 – 1.20)	0.96	(0.61 – 1.50)
p for trend			0.18		0.50
Clinical cutpoints of plasma 25(OH)D, nmol/L					
Deficient (<30)	47/80	1.09	(0.67 – 1.78)	1.00	(0.61 – 1.65)
Inadequate (30 to <50)	130/210	1.24	(0.88 – 1.76)	1.15	(0.81 – 1.64)
Adequate (50 – <75)	209/369	1.00	referent	1.00	referent
Adequate (≥ 75)	110/196	0.98	(0.69 – 1.39)	1.12	(0.76 – 1.55)
p for trend			0.74		0.65
Deficient/Inadequate (<50)	177/290	1.00	referent	1.00	referent
Adequate (≥ 50)	319/565	0.83	(0.62 – 1.11)	0.93	(0.69 – 1.25)
More than One Pathogenic Bacteria (n=630)					
Deficient/Inadequate (<50)	104/217	1.00	referent	1.00	referent
Adequate (≥ 50)	167/413	0.74	(0.53 – 1.03)	0.85	(0.60 – 1.19)
All orange complex bacteria [†] (n=400)					
Deficient/Inadequate (<50 nmol/L)	17/130	1.00	referent	1.00	referent
Adequate (≥ 50 nmol/L)	24/270	0.61	(0.32 – 1.17)	0.68	(0.37 – 1.32)
All red complex bacteria [†] (n=441)					
Deficient/Inadequate (<50)	33/146	1.00	referent	1.00	referent
Adequate (≥ 50)	49/295	0.68	(0.42 – 1.12)	0.80	(0.48–1.33)

* Adjusted for body mass index categorized as <25 kg/m², 25–30 kg/m², and >30 kg/m² and age

[†] Orange complex bacteria are *Fusobacterium nucleatum*, *Prevotella intermedia* and *Campylobacter rectus*. Red complex bacteria are *Porphyromonas gingivalis* and *Tannerella forsythensis*.

Table 4

Adjusted odds ratios (OR) and 95% confidence intervals (95% CIs) for prevalent pathogenic oral bacteria among women with adequate (>50 nmol/L) compared to deficient/inadequate (< 50 nmol/L) plasma 25(OH)D concentrations stratified by potential effect modifiers: the Osteoporosis and Periodontal Disease Study (1997–2000) (N=855)

Strata of potential effect modifier	# with outcome/ total #	Adjusted OR*	95% CI
Frequency of Flossing			
Once a week	154/245	0.61	(0.35 – 1.09)
> Once a week	146/247	1.11	(0.65 – 1.89)
Every day	195/360	1.13	(0.69 – 1.85)
p for interaction			0.22
Whole Mouth Mean PPD			
< 2mm	129/250	0.91	(0.53 – 1.57)
2 to < 3mm	348/570	0.91	(0.62 – 1.31)
3mm	19/33	1.84	(0.34 – 9.91)
p for interaction			0.34
Current Smoker			
No	475/829	1.00	(0.74 – 1.35)
Yes	21/26	0.25	(0.02 – 3.34)
p for interaction			0.029
Recent antibiotic Use			
No	458/785	0.97	(0.71 – 1.32)
Yes	38/70	0.59	(0.19 – 1.86)
p for interaction			0.54
Current Hormone therapy Use			
No	278/431	0.97	(0.65 – 1.45)
Yes	214/417	0.81	(0.53 – 1.24)
p for interaction			0.61
Self-reported diagnosis of diabetes			
No	475/816	0.92	(0.68 – 1.26)
Yes	21/39	0.63	(0.16 – 2.52)
p for interaction			0.69

* Adjusted for body mass index categorized as <25 kg/m², 25–30 kg/m², and >30 kg/m² and age