Relationship Between the Subgingival Microbiome and Menopausal Hormone Therapy Use: The Buffalo OsteoPerio Study.

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Summary: In a cross-sectional study of postmenopausal women, relative abundance of several subgingival microbiota differed significantly according to hormone therapy use, moreover, alpha diversity (Shannon index) and beta diversity differed significantly between current and never hormone therapy users.

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All authors made substantial contribution and have given final approval of the version of the manuscript to be published. MJL, KMH, CAA, MJB, YS, AEM, and JWW contributed to the conception, design, and data acquisition of the study. All authors have been involved with data analysis and interpretation. AIS, MJL, KMH, and JWW drafted the manuscript. All authors provided critical revision of the manuscript. MJL and JWW are responsible for the integrity of the data used in this manuscript.

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ABSTRACT

Background: This study investigated the association between menopausal hormone therapy (HT) use and the subgingival microbiome, for which published information is limited.

Methods: This cross-sectional study included 1,270 postmenopausal women, aged 53-81 years, who completed clinical examinations. Detailed information on HT use (type, delivery mode, duration) was obtained from questionnaires. HT use was categorized into three groups (never, former, current). 16S rRNA sequencing was performed on subgingival plaque samples obtained during dental examinations. Operational Taxonomic Units were centered log2-ratio (CLR) transformed to account for the compositional data structure. Analysis of variance was used to compare mean microbial relative abundances across HT categories with Benjamini-Hochberg correction.

Results: Significantly higher alpha diversity (Shannon Index) and beta diversity (Aitchison distance) was observed in never compared to current HT users ($P<0.05$, each). Of the total 245 microbial taxa identified, 18 taxa differed significantly among the three HT groups, 11 of which were higher in current users and 7 of which were lower in current users as compared to never users ($P<0.05$, each). Differences in relative abundance between never and current HT users were materially unchanged after adjustment for age, BMI, and oral hygiene.

Conclusion: Relative abundance of several subgingival bacteria differed significantly between never and current HT users in a cohort of postmenopausal women. Additional studies are needed to determine the extent that these relationships might account for the previously reported inverse association between HT use and periodontal disease in older women.

Key words: Postmenopause, Women, Periodontal disease, Hormones, Microbiota.

Introduction

Periodontitis is a chronic inflammatory disease, with serious health consequences including alveolar bone and tooth loss, and masticatory dysfunction affecting nutritional status, speech function and quality of life[1]. Periodontal disease is ranked 7th in prevalence globally, with 1.1 billion prevalent cases in 2019[2]. In the United States, 42% of adults 30 years or older have periodontitis, with the highest prevalence being 60% among individuals ≥65 years[3]. Periodontitis is associated with significant changes in the composition of the subgingival microbiome, with an observed dysbiotic state characterized by enrichment of disease-associated taxa such as *Tannerella forsythia* and *Treponema socranskii*, and depletion of health-associated taxa such as *Actinomyces naeslundii* and *Streptococcus sanguinis*[4].

The gingiva is a target tissue for progesterone and estrogen. Effects of estrogen on the periodontal tissues include stimulating proliferation of gingival fibroblasts, reducing T-cell mediated inflammation, and inhibiting polymorphonuclear leukocytes (PMNL) chemotaxis[5]. Changes in endogenous female sex hormones have been linked to several periodontal manifestations[5]. During puberty, increased secretion of female sex hormones is associated with gingival inflammation and increased prevalence of *Prevotella intermedia*[6]. During pregnancy, severity of gingival inflammation is correlated to female sex hormone levels, with observed reduction in inflammation following parturition[5]. Moreover, pathobionts identified in subgingival biofilms such as *Treponema denticola* and *P. intermedia,* were shown to be affected by female sex hormones in vitro[7,8].

After menopause, lower estrogen levels are hypothesized to affect periodontal disease through increased inflammation and alveolar bone resorption[9] and were found to be associated with higher gingival inflammation and clinical attachment loss during early menopause[10].

Few epidemiological studies have described the association between exogenous female sex hormones and the subgingival bacteria. Jensen et al. reported 16-fold difference in

Bacteroides species in women aged 18-40 using oral contraceptives versus non-users[11]. Klinger et al. found growth of subgingival *P. intermedia* can be affected by differences in estradiol dose and type of progestin in oral contraceptives in women aged 20-32[12]. Furthermore, Tarkkila et al. studied effects of menopausal hormone therapy (HT) in peri- and post-menopausal women aged 50-58 during 2-year follow-up. They compared subgingival plaque samples positive for certain periodontal bacteria using PCR and found HT users had significantly lower frequency of *Porphyromonas gingivalis* and *T. forsythia* than non-users[13]. However, these studies were of small sample size and used targeted methods to assess pre-specified microbes with limited ability to detect and characterize compositional diversity of the broader bacterial community.

The aim of our study was to investigate the relationship between history of HT use and the composition and diversity of the subgingival microbiome, measured using untargeted next generation sequencing methods, in a cohort of community dwelling postmenopausal women.

Materials and Methods

Study cohort

Our study included postmenopausal women, age 53-81 years, enrolled in the Buffalo Osteoporosis and Periodontitis (OsteoPerio) Study, an ancillary study of the Women's Health Initiative (WHI) observational study (WHI-OS) at the Buffalo, New York, clinical center. Details on WHI-OS and OsteoPerio study designs have been published[14–16]. Briefly, between 1993-1998, 2200 women were recruited into the WHI-OS at the Buffalo center. From 1997-2001, these women were then recruited into OsteoPerio ancillary study to assess the relationship between osteoporosis and periodontal disease. Comprehensive oral measures were made and samples of subgingival plaque were obtained and later sequenced for determination of the subgingival microbiome[16].

A total of 1,342 participated in the OsteoPerio study at baseline (1997-2001), of whom 1,270 had available data on subgingival plaque microbiome and information on HT use for the

present analysis. Questionnaires were used to obtain information on age, race and ethnicity, oral hygiene habits, medical history, and lifestyle habits including smoking history. Body mass index (BMI; kg/m^2) was calculated using height (cm) and weight (kg) measured in clinic using calibrated clinical scale and stadiometer. Neighborhood socioeconomic status (nSES) was characterized using questionnaires and census tract information to compute scores ranging from 0 to 100, with higher scores indicating more affluent tracts[17].

The University at Buffalo Institutional Review Board approved all study protocols, and written informed consent was obtained from participants. This manuscript followed the STROBE guidelines for human observational studies. Figure 1 shows flow chart of participant enrollment into the OsteoPerio study.

Hormone Therapy Use

Detailed information on hormone therapy use was obtained at OsteoPerio Study enrollment from questionnaires. Type (estrogen, estrogen + progestin), delivery mode (pills, transdermal patch) and duration of usage (years) were collected. For the primary analysis, women were categorized based on HT history into three groups (never, former, current).

Periodontal Assessment

Participants completed whole mouth dental examination conducted by trained dental examiners[15,16]. Decayed, missing, and filled teeth were recorded along with reason for missing teeth. For all teeth present (except third molars) probing measures were obtained including gingival bleeding on probing, pocket dept (PD) and clinical attachment level (CAL). Periodontal disease presence and severity was defined using criteria from the Centers for Disease Control and Prevention/American Academy of Periodontology (CDC/AAP)[18]. Participants were categorized into four groups: Mild (≥2 interproximal sites with ≥3 mm CAL and ≥2 interproximal sites with ≥4 mm PD (not on same tooth) or 1 site with ≥5 mm PD),

Moderate (≥2 interproximal sites with ≥4 mm CAL (not on same tooth) or ≥2 interproximal sites with ≥5 mm PD (not on same tooth)), Severe (≥2 interproximal sites with ≥6 mm CAL (not on same tooth) and ≥1 interproximal site with ≥5 mm PD), and None (no evidence of mild, moderate, or severe disease). As number of mild periodontal disease was low in our sample (1%), none and mild were combined for analyses. A new staging and grading classification of periodontitis has been published by the European Federation on Periodontitis (EFP)[19]. Because we did not systematically collect all information required for this new approach, we are not able to apply it in the present study. Findings from a recent study show good agreement between 2012 CDC/AAP and 2018 EFP approaches in classifying periodontitis presence and severity[20].

Subgingival plaque samples

Subgingival plaque samples were obtained at beginning of the oral exam by placing fine paper points into the gingival pockets of up to 12 pre-specified teeth (6 maxillary and 6 mandibular arch teeth) following a standardized protocol[21]. Paper points collected separately from upper and lower arches were then placed directly into 4 ml of lactated Ringer's solution. The subgingival plaque solution was then vortexed for dispersion of microorganisms, aliquoted into 0.5 ml straws, frozen immediately at -80^{o}C, and later placed in liquid nitrogen for long term storage[21].

Subgingival microbiome analysis

The procedures used to analyze the subgingival plaque microbiome have been published[16,22]. Briefly, metagenomic DNA was isolated from subgingival plaque samples using an automated system[#] and commercially available kit** with enzymatic pretreatment for efficient isolation of Gram-positive bacteria. After DNA purification, samples were eluted in a 96 well plate. Each plate had 85 to 88 subgingival plaque samples, duplicate of blank extraction negative controls, with subgingival plaque pools as a positive control. Extracted DNA was then quantified using commercial kits[††]. Bacterial 16S DNA was amplified using

16S V3 (341F) forward and V4 (805R) reverse primer pairs using commercial kits‡‡ as previously detailed[22]. The V3-V4 hypervariable region was selected based on findings from our previous study showing the shorter V3-V4 region provided more robust sequencing results than longer regions as V1-V3[22]. Sequencing was performed in the Genomics and Bioinformatics Core Laboratory at the State University of New York at Buffalo.

Polymerase chain reaction (PCR) amplifications and sequencing were performed on 96 samples at a time with both positive and negative controls (three to six plaque pools, one mock DNA, three extraction buffers, and one DNA-free water). To minimize batch effects, batches of 85 to 88 participant samples were processed together, randomly arranged on the 96-well plates with negative and positive controls. The plate controls were examined for each batch to satisfy quality of each plate.

Bioinformatics and Statistical analysis

Once paired-end reads were obtained, a custom Snakemake pipeline was used to identify and annotate operational taxonomic units (OTUs). The pipeline, which is publicly available at https://github.com/Wayne-Zen/SnaMP, first performs read quality filtering with FastX[23] and merges paired-end reads with the Paired-End reAd mergeR (PEAR)[24], and then identifies and annotates OTUs using BLAST[25] at 97% similarity against the Human Oral Microbiome Database (HOMD version 14.5)[26]. The pipeline produces an OTU abundance table and counts of reads passing each step for quality control. Sequences that did not match the database were discarded, and the raw OTU table was filtered to remove OTUs with read count less than 0.02% of total read count. As a quality control measure, rarefaction curves were used to determine a cutoff to remove samples containing < 3,000 sequence reads to ensure adequate sampling.

Study participants were characterized for descriptive purposes using means and standard deviations for continuous variables, or frequencies for categorical variables in the overall cohort and according to HT use (never, former, current). We applied a centered log2-ratio

(CLR) transformation on individual OTU relative abundance prior to further analysis. This transformation is recommended by Gloor et al. to account for complex compositional data structure, reduce likelihood of spurious correlations, and enhance meaningfulness of sub-composition comparisons[27]. CLR distribution of each OTU was approximately normal and variances in groups were similar by visual inspection. Positive CLR value for a given taxon indicates higher relative abundance compared to the overall composition geometric mean of zero, conversely, negative values indicate lower relative abundance. Moreover, fold differences for CLR values relative to the overall composition mean could be interpreted using 2 to the power of base 2 logarithm. For example, a CLR of three represents an 8-fold (2^3) higher abundance relative to the composition mean. Alpha diversity measures (bias-corrected Chao1 (richness), observed OTU count (richness), and Shannon entropy (evenness)) were used to assess species richness and evenness across HT use categories. Beta diversity was visualized using principal component analysis (PCA)[28]. The PCA was based on the Aitchison distance measure (Euclidean distances) between CLR transformed sample abundance vectors[29].

To evaluate differences in alpha diversity, we used ANOVA for normally distributed data and Kruskal–Wallis test for skewed data. Aitchison distance based PERMANOVA was used to evaluate differences in beta diversity.

The primary analysis including all OTUs was based on HT use categorized into three groups (never, former, current). ANOVA was used to compare CLR mean microbial abundances across HT use categories. We used Benjamini-Hochberg correction to account for multiple testing and control for false positive findings[30]. OTUs that showed a statistically significant difference across categories of HT use were further evaluated using Benjamini-Hochberg corrected post-hoc comparison to determine significant differences in microbial abundance between current and never HT groups. Because there was uncertainty regarding duration of use in the former HT group, the remainder of this analysis focused on current and never HT users. Microbiota that differed significantly between never and current HT groups were

analyzed using separate multivariable linear regressions wherein the dependent variable was CLR microbial abundance and HT use (never, current) was the independent variable. An unadjusted model and a model adjusting for age (years), BMI (kg/m^2), and frequency of teeth flossing were evaluated. Smoking and diabetes are known to influence subgingival microbiota, however, because their prevalence was low in our cohort (3.2% and 5%, respectively) they were not included in the analysis.

We explored potential effect modification of an association between HT use (never, current) and microbial abundances stratifying on CDC/AAP categories of periodontal disease (None/mild, moderate, severe), percent of sites with gingival bleeding (<30%, ≥30%), and oophorectomy status (no, yes). Because cell sizes reduced with stratification, tests of interaction had limited statistical power and therefore were not formally conducted. As such, differences between HT groups of sizeable magnitude across stratifying variables were noted by visual inspection.

To examine potential functional characteristics of the subgingival microbiota associated with HT use, we ran an in-silico PICRUSt analysis (version 1.1.1)[31]. Enrichment analysis was performed on level-3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[32]. Student's t-test with Benjamini-Hochberg correction for false discovery were used to compare current and never HT users on KEGG pathways. All statistical analyses reported herein were performed at a two-sided alpha of 0.05 using statistical software[§§].

Results

Characteristics of the 1,270 study participants overall and according to HT use categories are in Table 1. Overall, participants were predominately white (97%) with mean age of 66 years, mean BMI of 27 kg/m^2, and, on average, 23 teeth present. Prevalence of former and current HT use was 20% and 47%, respectively. There was low prevalence of self-reported diagnosed diabetes (5%), and current smoking (3%). According to CDC/AAP periodontal

disease categories, 25% had none/mild disease, 58% had moderate disease, and 16% had severe disease.

When considering participant characteristics according to HT use categories, current HT users were relatively younger (mean age 64 years), had higher prevalence of bilateral oophorectomy (23%), and higher none/mild CDC/AAP disease (28%). For current HT users, mean duration of use was 9.2 years, the majority reported taking oral HT pills (95%) with the remaining reported using skin patches (5%), 53% reported taking estrogen only formulations while 47% were taking estrogen + progestin.

Sequencing analysis identified a total of 245 microbial taxa in subgingival samples after filtering at 0.02% abundance, as previously described[33]. Figure 2 shows alpha and beta diversity for all OTUs according to HT use categories. No statistically significant differences in alpha diversity were found based on observed OTU counts (Figure 2A), and Chao1 index (Figure 2B). However, there was a statistically significant difference in Shannon index across HT use categories (uncorrected p=0.044), with pair-wise test showing never users had higher alpha diversity than current users (p=0.013) (Figure 2C). For beta diversity, there was a statistically significant difference between never and current users (PERMONA p=0.001) with no detectable difference in variance (PERMDISP p=0.078), although PCA plot inspection shows considerable overlap between the two groups (Figure 2D).

Of the total 245 bacterial taxa identified, there were 19 for which CLR mean abundance differed significantly (corrected P≤0.05) among HT use categories (Table 2). Pair-wise tests showed all but one OTU (*Prevotella sp.* Oral taxon 300; P=0.828) differed significantly (corrected P<0.05) between current and never HT users. Of the 18 bacteria that differed significantly between current and never users, 11 were in higher abundance in current users (*Streptococcus oralis, Streptococcus sanguinis, Rothia dentocariosa, Streptococcus intermedius, Actinomyces sp.* Oral taxon 169*, Actinomyces massiliensis, Corynebacterium durum, Veillonella rogosae, Actinomyces sp.* Oral taxon 171*, Haemophilus sp.* Oral taxon

036*,* and *Actinomyces sp.* Oral taxon 170), and 7 were in higher abundance in never users (TM7[G-1]sp. Oral taxon 349, *Treponema socranskii*, *Anaeroglobus geminatus*, *Tannerella forsythia*, *Veillonellaceae* [G-1] sp. Oral taxon 150, *Fretibacterium* sp. Oral taxon 359, and *Treponema maltophilum*). OTUs higher in current HT users belonged to phyla *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, while OTUs higher in HT never users belonged to phyla *Saccharibacteria*, *Spirochaetes*, *Firmicutes*, *Bacteroidetes*, *Synergistetes.* The four OTUs demonstrating the largest difference in CLR mean abundance between HT current and never users were *T. forsythia* (CLR mean: current 1.27 vs never 2.10), *F. sp.* Oral taxon 359 (current -0.36 vs never 0.47), *S. intermedius* (current 4.0 vs never 3.29), and TM7[G-1] sp. Oral taxon 349 (current 2.45 vs never 3.24). CLR mean abundances for the remaining 226 OTUs not significantly different across HT use categories (see Table S1 in online Journal of Periodontology).

Table 3 shows results of the linear regression models for the 18 OTUs that differed between HT current and never users. After adjustment for age, BMI and teeth flossing, observed differences remained appreciably unchanged in 2 OTUs (TM7[G-1] sp. Oral taxon 349, and *F. sp*. Oral taxon 359), while differences in the 16 remaining OTUs were attenuated. For 12 OTUs (*S. oralis, R. dentocariosa, S. intermedius,* TM7[G-1] sp. Oral taxon 349, *T. forsythia, A. sp.* Oral taxon 169, *A. massiliensis, V. rogosae, A. sp.* Oral taxon 171, *F.* sp. Oral taxon 359, *T. maltophilum, A. sp.* Oral taxon 170) differences between the two HT groups remained statistically significant, while for 6 OTUs (*S. sanguinis*, *T. socranskii, A. geminatus, V.* [G-1] sp. Oral taxon 150, *C. durum*, *H. sp.* Oral taxon 036) differences were not statistically significant after controlling for age, BMI and teeth flossing.

Table 4 shows results of the linear regression models stratified by CDC/AAP categories. For the unadjusted model, differences between HT groups were of similar direction for the 18 OTUs across CDC/AAP categories. However, none/mild category had an appreciably larger CLR mean difference for 3 OTUs (*R. dentocariosa, V.* [G-1] sp. Oral taxon 150, and *C. durum*), the moderate category had an appreciably larger difference for 1 OTU (*A.*

geminatus), and the severe category had an appreciably larger difference for 8 OTUs (*S. intermedius, T. socranskii*, *T. forsythia*, *A.* sp. Oral taxon 169, *F.* sp. Oral taxon 359, *T. maltophilum*, *H.* sp. Oral taxon 036, and *A.* sp. Oral taxon 170). For the majority of bacteria, the pattern in differences between HT never and current users remained consistent following adjustment for age, BMI and teeth flossing, with some exceptions where attenuation was more pronounced, such as for *R. dentocariosa*, *T. socranskii, A. geminatus,* and *C. durum*.

Of the 18 OTUs that differed significantly between current and never users, we conducted further exploratory analysis within current users according to HT formulation (estrogen vs estrogen + progestin) and median duration of use (<8 vs ≥8 years), oophorectomy status (no vs yes), and percent of sites with gingival bleeding (<30% vs ≥30%). There was no statistically significant difference in bacterial abundance according to formulation or median duration of use (see Tables S2 and S3 in online Journal of Periodontology), oophorectomy status, though small sample size limited this analysis (see Table S4 in online Journal of Periodontology) or gingival bleeding (see Table S5 in online Journal of Periodontology).

Results of in-silico PICRUSt analysis resulted in 35 pathways that differed significantly between HT current and never users (corrected $P<0.05$). Of these, 9 functional pathways were more evident and 26 pathways less evident for bacteria among HT current users than never users. Enriched bacterial functional pathways included cellular adhesion and signaling, and carbohydrate metabolism, whereas less enriched pathways included oxidative glycolysis, lipid metabolism, and bacterial chemotaxis. See Figure S1 in online Journal of Periodontology for complete findings from the PICRUSt analysis.

Discussion

The objective of our study was to investigate the association between history of HT use and the composition and diversity of the subgingival microbiome in postmenopausal women. We used data from next generation sequencing and found 19 bacterial taxa differed significantly across HT use categories, of which 18 differed significantly between current and never HT

users. Furthermore, there was a statistically significant difference in beta diversity and alpha diversity (Shannon index) between never and current HT users.

Several periodontal changes in women have been shown to be associated with changes in both endogenous and exogenous female sex hormones[34]. It is thought these changes may be explained by direct effect of estrogen and/or progesterone on the periodontium as a target tissue[5]. Genco and Grossi proposed a biological model by which lower estrogen levels could contribute to periodontal disease including up-regulation of monocytes and macrophages, with subsequent increase in pro-inflammatory cytokines, leading to connective tissue destruction and alveolar bone resorption[9]. A randomized clinical trial showed HT significantly increased alveolar bone mass compared with placebo[35]. Moreover, others have pointed to a possible direct effect of female sex hormones on the composition of periodontal bacteria[36]. However, most studies on exogenous female sex hormone and oral bacteria were conducted on pre-menopausal women and investigated oral contraceptives, which have relatively higher concentrations of estrogen and progestin than menopausal HT[11,12]. To our knowledge, our study is the first to investigate this relation in postmenopausal women using high-throughput sequencing to better characterize the subgingival microbiome.

Previous studies have shown periodontitis is characterized by increased diversity of microbial communities[4]. Griffen et al. found alpha and beta diversity to be higher in those with chronic periodontitis versus healthy controls[37]. Genco et al. used the OsteoPerio cohort and reported higher alpha and beta diversity in severe compared to none/mild periodontal disease defined using CDC/AAP criteria[33]. This is similar to other pathological conditions as bacterial vaginosis, where the vaginal microbiome shows higher diversity in diseased states[38,39]. In our study, HT never users had significantly higher alpha (Shannon index) and beta diversity than current users.

At the phyla level, HT current users had higher abundance of OTUs belonging to the phyla *Actinobacteria*, *Firmicutes* and *Proteobacteria.* Previous bioinformatic analysis suggested

these phyla might express genes for steroid hormone metabolism[40,41]. Results in the present study based on PICRUSt analysis at the species level did not reveal higher enrichment of pathways involving steroid metabolism in current HT users than never users. Bacterial functional pathways involved with carbohydrate metabolism and cellular adhesion were significantly enriched in HT current users, whereas pathways involving oxidative glycolysis, lipid metabolism, and chemotaxis were less enriched in HT current users. While in-silico PICRUSt provides an ability to speculate about bacterial functional pathways, studies that directly measure functional expression of bacterial genes, such as transcriptomics, are needed to definitively characterize bacterial function in relation to exogenous menopausal hormone use. Another alternative explanation is that observed difference between HT current and never users is due to increased enrichment of subgingival pathobionts acquired with increased inflammation and longer-term alveolar bone loss that is associated with decreasing endogenous estrogen levels during menopause.

Current HT use was associated with significantly lower abundance of *T. forsythia* and *T. socranskii*, pathobionts known to be associated with periodontitis[42,43], as well as several pathobionts (TM7[G-1] sp. Oral taxon 349, *A.geminatus*, *V.* [G-1] sp. Oral taxon 150, *F.* sp. Oral taxon 359, and *T. maltophilum*) previously shown in this cohort to be elevated in severe and moderate periodontal disease[33]. Moreover, current HT use was associated with higher abundance of bacteria associated with mild periodontal disease such as *S. oralis* and *A. massiliensis*[33]. Previously, HT use was found to be associated with lower *T. Forsythia* in subgingival plaque samples among peri- and post-menopausal women in a 2-year prospective study by Tarkkila et al.[13]. However, their study was of smaller sample size and used targeted PCR to test a limited number of bacteria.

The strengths and limitations of our study should be considered when interpreting its findings. Strengths includes using a large sample of postmenopausal women whose selection into the study was not based on periodontal disease allowing more generalizability of findings. Also, use of untargeted next generation sequencing techniques provided an

opportunity to identify more subgingival bacteria compared to previous targeted methods, and adjustment for potential confounders such as age and BMI. Limitations includes cross-sectional design, which does not establish temporality and precludes causal inference between HT use and periodontal microbiome composition, and potential for residual confounding given the observational nature of the data. Because the OsteoPerio Study is ancillary to the Women's Health Initiative which did not include men, our findings are restricted to women. Taxonomic OTU annotation was completed using HOMD version 14.5, which could result in an incomplete characterization of microbiota as additional taxa are added to future versions. At present, the impact that long-term sample storage at -80^{o}C has on results while not entirely clear, is thought to be less concerning with DNA-based analyses as 16S sequencing[44]. Last, complete information on measured endogenous hormone concentrations were not available for consideration in this analysis.

Conclusions

In our cross-sectional study, HT use was associated with favorable periodontal profile in terms of lower abundance of bacteria like *T. forsythia* and *T. socranskii, known pathobionts for more severe periodontal disease.* Hormone therapy was also associated with lower microbial diversity. Prospective studies are needed where change in menopausal HT use can be characterized and then related with the composition and diversity of the subgingival microbiome in women.

Footnotes

QIAsymphony SP, Qiagen, Valencia, CA, USA.

** QIAsymphony DSP Virus/Pathogen Mini Kit, Qiagen, Valencia, CA, USA.

†† Quant-iT™ High-Sensitivity dsDNA Assay Kit, Invitrogen, USA.

‡‡ MiSeq reagent kit V3 2x300, Illumina, CA, USA.

§§ SAS v.9.4, SAS Institute, Cary, NC, USA. and R v4.1.0, R foundation, Vienna, Austria.

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Conflictions of Interests: The authors have no conflicts of interest or relevant disclosures.

Availability of data and materials: Data that support the findings of this study are available from the authors upon reasonable request and with permission of the U.S. Women's Health Initiative program.

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Figures

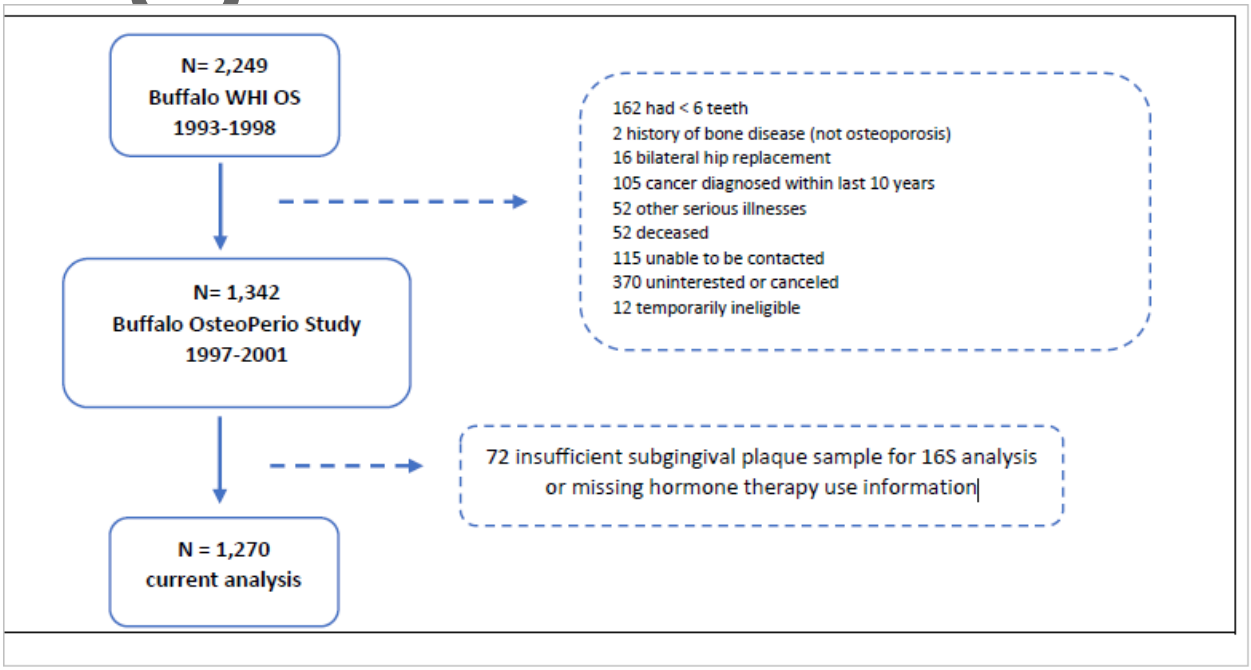

Figure 1. Flowchart of participants into the Buffalo OsteoPerio study cohort. Adapted from Banack et al. (2018).

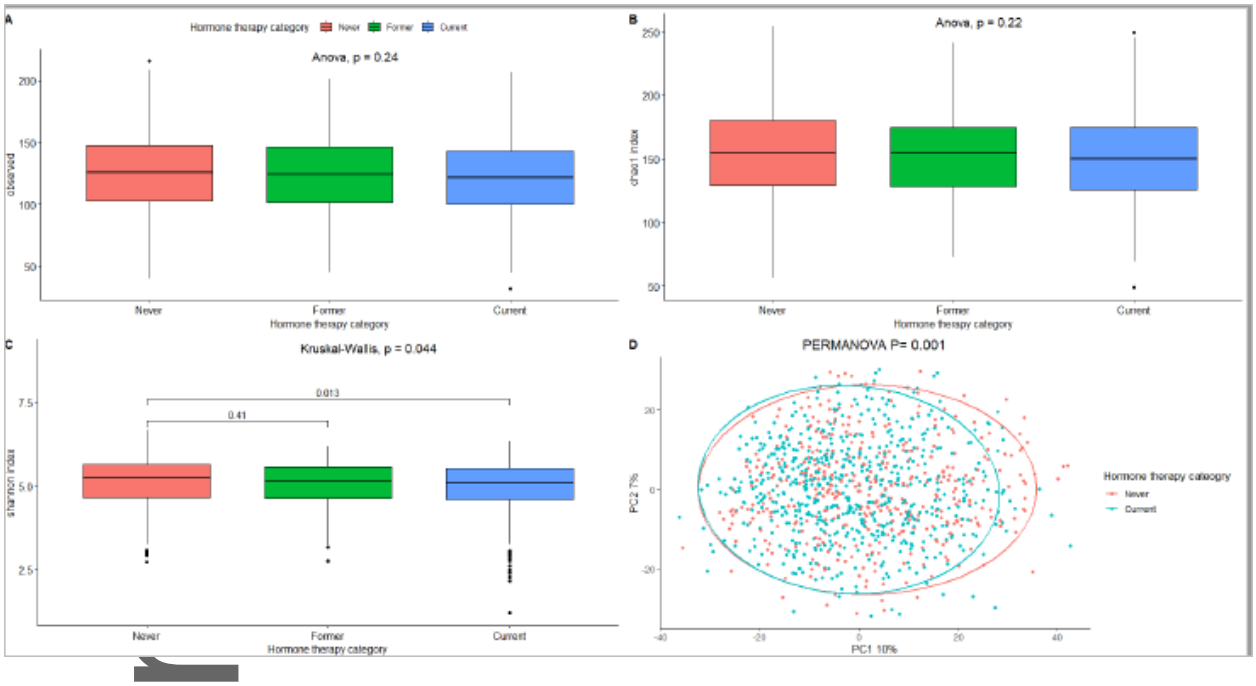

Figure 2. Alpha and beta diversity according to Hormone therapy use categories. **A** alpha diversity (observed OTU count), **B** alpha diversity (Chao1 index), **C** alpha diversity (Shannon index), **D** principal component analysis plot for beta diversity using Aitchison distance. PC: principal component.

Tables

Table 1. Participant characteristics overall and according to menopausal HT use. Data are mean (SD) or N (%).

Table 2. Subgingival bacteria (n = 19) that differed significantly according to menopausal HT Use. Data are CLR mean (SD) OTU.

Table 3. Linear regression for CLR of Subgingival bacteria (n = 18) that differed significantly between never and current HT Use adjusted by age, BMI, and teeth flossing.

Table 4. Subgingival bacteria (n = 18) that differed significantly between never and current HT use stratified by CDC/AAP categories.

Table 1. Participant characteristics overall and according to menopausal HT use. Data are mean (SD) or N (%).

Characteristic	Overall	History of Hormone therapy use		
		Never	Former	Current*
	(n = 1270)	(n = 409)	(n = 255)	(n = 606)
Age (years)	66.2 (7.0)	68.1 (7.0)	67.3 (7.2)	64.4 (6.6)
BMI (kg/m2)	26.6 (5.1)	27.1 (5.4)	27.1 (5.6)	26.1 (4.7)
Neighborhood SES	76.1 (7.0)	75.2 (7.2)	76.1 (7.2)	76.8 (6.7)
Missing N (%)	2 (0.2%)			
Number of teeth present at examination	23.2 (5.3)	22.4 (5.9)	23.4 (5.1)	23.7 (4.9)
Years since menopause	16.8 (8.6)	17.8 (8.4)	18.3 (9.4)	15.5 (8.2)
Missing N (%)	37 (2.9%)			
Percent of sites with gingival bleeding	35 (23)	37 (23)	34 (24)	33 (23)
Missing N (%)	7 (0.6%)			
Race-ethnicity: White	1236 (97.3%)	399 (97.6 %)	243 (95.3 %)	594 (98.0 %)
CDC/AAP periodontal disease				
None/Mild	315 (24.8%)	94 (23.4 %)	53 (21.0 %)	168 (27.9 %)
Moderate	734 (57.8%)	241 (60.1 %)	153 (60.5 %)	340 (56.5 %)
Severe	207 (16.3%)	66 (16.5 %)	47 (18.6 %)	94 (15.6 %)
Missing	14 (1.1%)			
Bilateral oophorectomy: Yes	209 (16.5%)	38 (9.4 %)	37 (14.7 %)	134 (22.5 %)
Smoking status				
Never	671 (52.8%)	232 (56.7 %)	125 (49.0 %)	314 (51.9 %)
Former	558 (43.9%)	164 (40.1 %)	115 (45.1 %)	279 (46.1 %)
Current	40 (3.2%)	13 (3.2 %)	15 (5.9 %)	12 (2.0 %)
Missing	1 (0.1%)			

History of diabetes treated with medication: Yes	64 (5.0 %)	24 (5.9 %)	17 (6.7 %)	23 (3.8 %)
Frequency of teeth flossing				
Everyday	550 (43.3%)	179 (43.9%)	112 (44.3%)	259 (43.0%)

*Formulation (319 (52.6%) estrogen alone, 287 (47.4%) estrogen + progestin), dosage form (577 (95.2%) pills, 29 (4.8%) transdermal patch), duration of use (mean: 9.2 years, standard deviation: 7.8).

Table 2. Subgingival bacteria (n = 19) that differed significantly according to menopausal HT Use. Data are CLR mean (SD) OTU.

OTU label (species level annotation)	Hormone Therapy Use			P value	
	Never (n=409)	Former (n=255)	Current (n =606)	Across the three HT groups *	Current vs never †
Streptococcus oralis ‡	7.58 (1.95)	7.87 (1.71)	7.99 (1.88)	0.050	0.001
Streptococcus sanguinis	4.38 (2.76)	4.58 (2.72)	4.99 (2.38)	0.028	0.001
Rothia dentocariosa	3.70 (3.09)	3.88 (3.02)	4.39 (2.95)	0.028	0.001
Streptococcus intermedius	3.29 (3.32)	3.41 (3.55)	4.10 (3.23)	0.019	0.001
TM7 [G-1] sp. oral taxon 349 ‡	3.24 (3.27)	2.81 (3.49)	2.45 (3.48)	0.032	0.001
Treponema socranskii ‡	2.15 (2.62)	2.34 (2.52)	1.76 (2.55)	0.050	0.018
Anaeroglobus geminatus ‡	2.15 (3.58)	2.33 (3.50)	1.45 (3.46)	0.025	0.003
Tannerella forsythia ‡	2.10 (3.44)	2.08 (3.41)	1.27 (3.39)	0.019	0.001
Actinomyces sp. oral taxon 169	1.30 (3.13)	1.55 (3.21)	2.00 (3.01)	0.032	0.001
Prevotella sp. oral taxon 300	1.09 (2.92)	1.77 (3.00)	1.05 (3.01)	0.050	0.828
Actinomyces massiliensis ‡	0.85 (2.46)	0.94 (2.52)	1.44 (2.31)	0.019	0.001
Veillonellaceae [G-1] sp. oral taxon 150 ‡	1.30 (2.99)	1.19 (3.15)	0.68 (3.20)	0.050	0.003
Corynebacterium durum	0.55 (2.91)	0.32 (2.89)	1.00 (2.84)	0.039	0.018
Veillonella rogosae	0.12 (3.25)	0.69 (3.13)	0.78 (3.15)	0.050	0.002
Actinomyces sp. oral taxon 171	0.12 (2.76)	0.21 (2.74)	0.70 (2.70)	0.034	0.001
Fretibacterium sp. oral taxon 359 ‡	0.47 (3.92)	-0.18 (3.72)	-0.36 (3.57)	0.039	0.001
Treponema maltophilum ‡	-0.38 (2.70)	-0.73 (2.66)	-0.98 (2.53)	0.039	0.001
Haemophilus sp. oral taxon 036	-1.39 (3.04)	-1.74 (2.83)	-0.92 (3.22)	0.028	0.020
Actinomyces sp. oral taxon 170	-1.75 (2.85)	-1.92 (2.74)	-1.18 (3.11)	0.025	0.003

* Significant P-value from ANOVA F-test across the three HT categories after Benjamini-Hochberg correction.

† Difference between current and never users after Benjamini-Hochberg correction.

‡ Previously shown in the OsetoPerio cohort to be associated with CDC/AAP periodontal categories (Genco et al. 2019)

Table 3. Linear regression for CLR of Subgingival bacteria (n = 18) that differed significantly between never and current HT Use adjusted by age, BMI, and teeth flossing.

OTU label (species level annotation)	Unadjusted			Adjusted for Age, BMI, and teeth flossing		
	LS* mean Never (n=409) / LS* mean Current (n =606)	B† (SE‡)	P§	LS* mean Never (n=409) / LS* mean Current (n =606)	β† (SE‡)	P§
Streptococcus oralis	7.58 / 7.99	0.41 (0.12)	0.001	7.53 / 7.85	0.32 (0.13)	0.012
Streptococcus sanguinis	4.38 / 4.99	0.61 (0.16)	<0.001	4.47 / 4.78	0.31 (0.17)	0.061
Rothia dentocariosa	3.70 / 4.39	0.69 (0.19)	<0.001	3.65 / 4.17	0.52 (0.20)	0.008
Streptococcus intermedius	3.29 / 4.10	0.81 (0.21)	<0.001	3.28 / 3.98	0.70 (0.22)	0.001
TM7_[G-1] sp._oral_taxon_349	3.24 / 2.45	-0.80 (0.22)	<0.001	3.31 / 2.57	-0.73 (0.23)	0.001
Treponema socranskii	2.15 / 1.76	-0.39 (0.16)	0.017	2.12 / 1.94	-0.18 (0.17)	0.284
Anaeroglobus geminatus	2.15 / 1.45	-0.70 (0.22)	0.002	2.04 / 1.63	-0.41 (0.23)	0.074
Tannerella forsythia	2.10 / 1.27	-0.82 (0.22)	<0.001	2.00 / 1.27	-0.73 (0.23)	0.001
Actinomyces sp._oral_taxon_169	1.30 / 2.00	0.71 (0.20)	<0.001	1.31 / 1.89	0.58 (0.20)	0.005
Actinomyces massiliensis	0.85	0.59	<0.001	0.91	0.40	0.011

	1.44	(0.15)		1.31	(0.16)	
Veillonellaceae_[G-1] sp._oral_taxon_150	1.30 0.68	-0.62 (0.20)	0.002	1.27 0.93	-0.34 (0.21)	0.096
Corynebacterium durum	0.55 1.00	0.45 (0.18)	0.015	0.57 0.69	0.12 (0.19)	0.536
Veillonella rogosae	0.12 0.78	0.66 (0.20)	0.001	0.09 0.51	0.42 (0.21)	0.047
Actinomyces sp._oral_taxon_171	0.12 0.70	0.59 (0.17)	0.001	0.11 0.57	0.45 (0.18)	0.013
Fretibacterium sp._oral_taxon_359	0.47 -0.36	-0.82 (0.24)	0.001	0.49 -0.35	-0.84 (0.25)	0.001
Treponema maltophilum	-0.38 -0.98	-0.59 (0.17)	<0.001	-0.34 -0.82	-0.47 (0.17)	0.006
Haemophilus sp._oral_taxon_036	-1.39 -0.92	0.47 (0.20)	0.021	-1.58 -1.22	0.36 (0.21)	0.088
Actinomyces sp._oral_taxon_170	-1.75 -1.18	0.57 (0.19)	0.003	-1.77 -1.37	0.40 (0.20)	0.047

* Least square mean in the linear regression model.

† β coefficients represents least square mean difference for HT current vs never users in the linear regression model.

‡ Standard error for β coefficients.

§Uncorrected p value for β coefficients of mean difference for HT current vs never users.

Table 4. Subgingival bacteria (n = 18) that differed significantly between never and current HT use stratified by CDC/AAP categories.

OTU label (species level annotation)	CDC/AAP None/Mild N = 262			CDC/AAP Moderate N= 581			CDC/AAP Severe N= 160		
	LS* mean Never (n=94)	β† (SE‡)	β§ (SE‡)	LS* mean Never (n=241)	β† (SE‡)	β§ (SE‡)	LS* mean Never (n=66)	β† (SE‡)	β§ (SE‡)
	LS* mean Current (n=168)			LS* mean Current (n=340)			LS* mean Current (n=94)		
Streptococcus oralis	7.77 8.15	0.38 (0.24)	0.19 (0.25)	7.69 8.12	**0.44** (0.16)	**0.37** (0.16)	6.98 7.25	0.27 (0.32)	0.21 (0.33)
Streptococcus sanguinis	4.83 5.38	0.55 (0.32)	0.24 (0.33)	4.42 4.97	**0.55** (0.21)	0.27 (0.21)	3.77 4.46	0.69 (0.43)	0.37 (0.44)
Rothia dentocariosa	3.55 4.67	**1.11** (0.35)	**0.91** (0.37)	4.01 4.51	**0.50** (0.25)	0.42 (0.26)	3.03 3.49	0.46 (0.54)	0.01 (0.56)
Streptococcus intermedius	3.72 4.34	0.63 (0.40)	0.65 (0.42)	3.46 4.20	**0.74** (0.27)	**0.65** (0.28)	2.18 3.20	1.02 (0.55)	0.62 (0.58)
TM7 [G-1] sp. oral taxon 349	2.76 1.83	**-0.93** (0.45)	-0.71 (0.47)	3.19 2.48	**-0.71** (0.28)	**-0.77** (0.30)	4.14 3.38	-0.75 (0.52)	-0.37 (0.55)
Treponema socranskii	1.19 1.08	-0.11 (0.32)	0.17 (0.33)	2.17 1.81	-0.36 (0.21)	-0.17 (0.22)	3.42 2.72	-0.70 (0.40)	-0.47 (0.42)
Anaeroglobus geminatus	1.38 1.03	-0.35 (0.45)	0.15 (0.47)	2.16 1.37	**-0.79** (0.30)	-0.55 (0.31)	3.03 2.57	-0.47 (0.51)	-0.35 (0.54)
Tannerella forsythia	1.05 0.14	**-0.90** (0.39)	-0.78 (0.41)	1.89 1.37	-0.52 (0.29)	-0.44 (0.30)	4.37 2.86	**-1.51** (0.53)	**-1.3** (0.55)
Actinomyces sp.	1.75	0.23	0.07	1.40	**0.75**	**0.66**	0.34	**1.16**	0.88

oral taxon 169	1.99	(0.39)	(0.41)	2.15	(0.26)	(0.27)	1.50	(0.49)	(0.51)
Actinomyces massiliensis	1.12	**0.64**	0.27	0.99	**0.49**	0.33	0.10	0.70	0.62
	1.76	(0.30)	(0.31)	1.48	(0.20)	(0.20)	0.79	(0.37)	(0.40)
Veillonellaceae [G-1] sp. oral taxon 150	0.81	**-0.88**	-0.48	1.21	**-0.53**	-0.30	2.25	-0.41	-0.24
	-0.07	(0.39)	(0.41)	0.68	(0.26)	(0.27)	1.83	(0.46)	(0.49)
Corynebacterium durum	0.60	**0.86**	0.38	0.73	0.18	-0.13	-0.08	0.63	0.32
	1.46	(0.36)	(0.36)	0.91	(0.24)	(0.25)	0.54	(0.46)	(0.49)
Veillonella rogosae	0.37	0.51	0.05	0.16	**0.69**	0.44	-0.11	0.51	0.47
	0.88	(0.41)	(0.43)	0.85	(0.27)	(0.28)	0.41	(0.50)	(0.53)
Actinomyces sp. oral taxon 171	-0.13	**1.10**	**0.93**	0.44	0.24	0.13	-0.61	**0.94**	0.68
	0.97	(0.34)	(0.35)	0.68	(0.23)	(0.24)	0.33	(0.44)	(0.47)
Fretibacterium sp. oral taxon 359	-0.51	**-0.84**	**-0.89**	0.13	-0.45	-0.47	2.99	**-1.66**	**-1.45**
	-1.35	(0.38)	(0.40)	-0.32	(0.31)	(0.32)	1.33	(0.69)	(0.72)
Treponema maltophilum	-1.19	-0.43	-0.34	-0.50	**-0.47**	-0.34	1.23	**-1.11**	**-1.05**
	-1.62	(0.28)	(0.29)	-0.97	(0.21)	(0.22)	0.12	(0.49)	(0.52)
Haemophilus sp. oral taxon 036	-0.71	0.22	0.11	-1.35	0.33	0.25	-2.71	**1.44**	**1.15**
	-0.49	(0.42)	(0.44)	-1.02	(0.26)	(0.27)	-1.27	(0.47)	(0.49)
Actinomyces sp. oral taxon 170	-1.32	0.51	0.13	-1.72	0.41	0.29	-2.47	**1.14**	0.95
	-0.81	(0.39)	(0.41)	-1.31	(0.25)	(0.26)	-1.33	(0.48)	(0.51)

Bold text indicates uncorrected p value (≤ 0.05) from for β coefficients of mean difference for HT current vs never users.

* Least square mean in the linear regression model.

†β coefficients represents least square mean difference for HT current vs never users in the linear regression model.

‡ Standard error for β coefficients.

§ linear regression model adjusted for age, BMI, and teeth flossing.