

4-1-2022

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Longitudinal analysis of the impact of oral contraceptive use on the gut microbiome

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Abstract

Introduction. Evidence has linked exogenous and endogenous sex hormones with the human microbiome.

Hypothesis/Gap statement. The longitudinal effects of oral contraceptives (OC) on the human gut microbiome have not previously been studied.

Aim. We sought to examine the longitudinal impact of OC use on the taxonomic composition and metabolic functions of the gut microbiota and endogenous sex steroid hormones after initiation of OC use.

Methodology. We recruited ten healthy women who provided blood and stool samples prior to OC use, 1 month and 6 months after starting OC. We measured serum levels of sex hormones, including estradiol, progesterone, sex hormone-binding globulin (SHBG), and total testosterone. Shotgun metagenomic sequencing was performed on DNA extracted from faecal samples. Species and metabolic pathway abundances were determined using MetaPhlAn2 and HUMAnN2. Multivariate association with linear models was used to identify microbial species and metabolic pathways associated with OC use and endogenous levels of sex hormones.

Results. The percentage variance of the microbial community explained by individual factors ranged from 9.9% for age to 2.7% for time since initiation of OC use. We observed no changes in the diversity or composition of the gut microbiome following OC initiation. However, the relative abundance of the biosynthesis pathways of peptidoglycan, amino acids (lysine, threonine, methionine, and tryptophan), and the NAD salvage pathway increased after OC initiation. In addition, serum levels of estradiol and SHBG were positively associated with *Eubacterium ramulus*, a flavonoid-degrading bacterium. Similarly, microbes involving biosynthesis of L-lysine, L-threonine, and L-methionine were significantly associated with lower estradiol, SHBG, and higher levels of total testosterone.

Conclusion. Our study provides the first piece of evidence supporting the association between exogenous and endogenous sex hormones and gut microbiome composition and function.

Received 04 October 2021; Accepted 14 January 2022; Published 22 April 2022

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Keywords: human gut microbiome; shotgun metagenomic sequencing; oral contraceptive; sex hormone; estradiol; sex hormone-binding globulin.

Abbreviations: FDR, false discovery rate; HMP, Human Microbiome Project; HUMAnN, HMP Unified Metabolic Analysis Network; MaAsLin, multivariable linear mixed model; MetaPhlAn, metagenomic phylogenetic analysis; MGH, Massachusetts General Hospital; OC, oral contraceptive; PERMANOVA, permutational multivariate analysis of variance; SHBG, sex hormone-binding globulin.

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Two supplementary figures and three supplementary tables are available with the online version of this article.

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INTRODUCTION

Exogenous hormone use in the form of oral contraceptives (OC) has been previously linked to the development and progression of several immune-mediated diseases, such as Crohn's disease [1, 2], rheumatoid arthritis [3], and systemic lupus erythematosus [4–6]. Nevertheless, the exact mechanism underlying these observations has not been fully established. [7]

Some evidence has suggested a link between exogenous and endogenous sex hormones and the human microbiome. Specifically, OC use was shown to be associated with a higher prevalence of certain *Candida* and *Prevotella* species in periodontal pockets, as well as an increased risk of severe periodontitis [8]. Similarly, exogenous hormone use in postmenopausal women is associated with changes in vaginal microbial composition, with an increase in lactobacillus species and a decrease in urogenital infections [9–12]. Furthermore, in a mouse model of type one diabetes, transferring the gut microbiota from adult males to immature females led to increased testosterone levels and reduced risk of developing type one diabetes [13]. Lastly, a cross-sectional study observed a positive correlation between serum levels of estradiol and the diversity and composition of gut microbial communities in women [14].

Despite the evidence for the impact of both endogenous and exogenous hormones on the gut microbiome, no prior study has systematically evaluated the effect of initiating OC on the community structure and function of the gut microbiome. We, therefore, sought to examine the longitudinal impact of OC use on the taxonomic composition and metabolic functions of the gut microbiota and endogenous sex steroid hormones in healthy women followed for 6 months after initiation of OC use.

METHODS

Study population and sample collection

Our study recruited ten healthy premenopausal women aged 16–40 years from the Massachusetts General Hospital (MGH). Participants started OC use between January 2015 and August 2018. They provided blood and stool samples at three time points: baseline prior to initiation of OC use, one, and 6 months after initiation of OC use (Fig. 1). In addition, participants completed a detailed questionnaire to assess lifestyle factors such as diet (such as consumption and frequency of dairy products, fruit, vegetables, red and processed meat and alcoholic drinks) and medication use (such as antibiotics, probiotics, and use of non-steroidal anti-inflammatory drugs) at study entry. To account for the influence of normal physiologic changes on sex hormone measurements, blood samples from each individual were collected at the follicular stage (3–5 days after the beginning of the menstrual cycle) for each study visit using serum-separating tubes and stored in -80°C freezers until further analysis. Participants also self-collected stool samples within 48 h of blood draw with the exception of one timepoint when the participant provided stool sample 3 weeks later using a collection vial containing RNA stabiliser *RNAlater* (Invitrogen, Fisher). Stool samples were handled at ambient temperature and shipped overnight to MGH. Upon arrival, samples were stored in -80°C freezers immediately until nucleic acid extraction. The stool self-collection method was previously validated in the Human Microbiome Project (HMP) [15]. This study was approved by the Institutional Review Board at Mass General Brigham (Protocol # 2013P002125). Informed consent was obtained from all participants.

Serum sex hormone measurements

Serum samples were shipped to the Mayo Clinic on dry ice to measure circulating hormones, including estradiol, progesterone, sex hormone-binding globulin (SHBG), and total testosterone. We measured estradiol and total testosterone using liquid chromatography-tandem mass spectrometry, progesterone using electrochemiluminescence immunoassay, and SHBG using solid-phase, two-site chemiluminescent immunoassay. All measurements were done in a single batch.

Metagenomic sequencing and processing

DNA was extracted from the stool samples preserved in *RNAlater* using a previously established protocol [16]. We used NEBNext DNA Library Preparation Kit for library construction. Metagenomic sequencing was performed at the MGH NextGen Sequencing Core. We used the Illumina HiSeq2500 pair-ended (2×101 nucleotides) platform for the whole metagenome shotgun sequencing. Raw sequencing reads were processed using the KneadData pipeline version v0.7.10 (<http://huttenhower.sph.harvard.edu/kneaddata>) with default parameters to trim short reads ($<50\%$ of total input read length) and remove sequences of human origin. The sequencing depth (mean \pm standard deviation) before and after quality control using KneadData was 1.8 ± 0.3 and 1.6 ± 0.3 giga-nucleotides (Gnt), respectively. Metagenomic sequencing and processing of all samples were performed in a single batch.

Taxonomic and functional profiling

Taxonomic and functional profiling were conducted by applying the bioBakery meta'omics workflow according to the HMP protocol [17]. We used the Metagenomic Phylogenetic Analysis tool (MetaPhlAn version 2.7.7) for taxonomic profiling [18]. MetaPhlAn classifies metagenomic reads to taxonomies and generates the relative abundances of microbial species in each sample based on approximately one million clade-specific marker genes derived from 17000 microbial genomes (corresponding

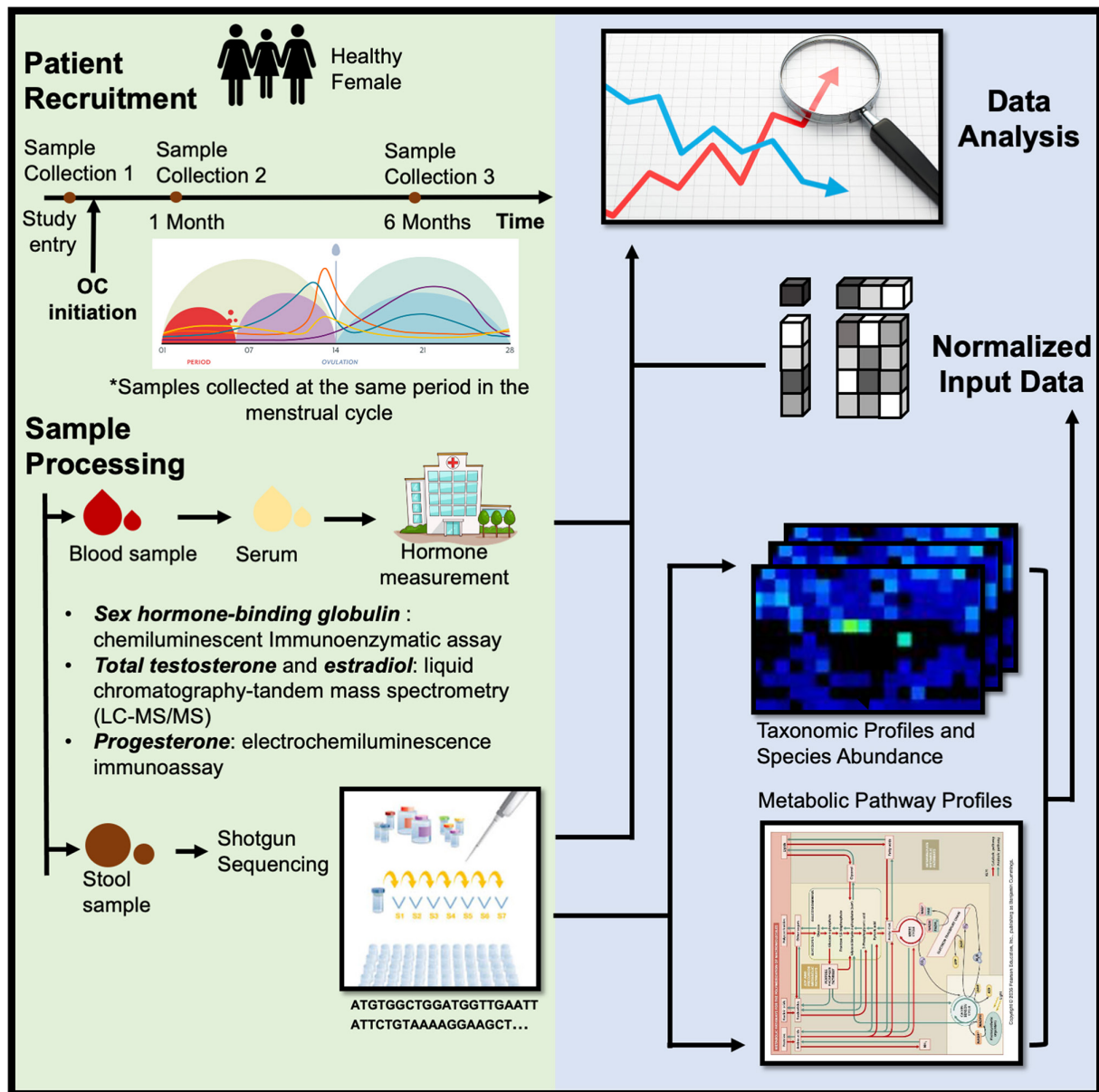


Fig. 1. Study design. We recruited ten healthy premenopausal women aged 16–40 years from the Massachusetts General Hospital. At study entry, each participant completed a detailed questionnaire to assess medical and lifestyle factors, such as diet, medical history, and medications use. Participants started OC use between January 2015 and August 2018. They also provided blood and stool samples at three time points: baseline prior to initiation of OC use, one, and 6 months after initiation of OC use.

to >13500 bacterial and archaeal). Microbial species that did not surpass the minimum prevalence (10% of samples) and relative abundance (0.01%) threshold were excluded from analyses.

We performed metagenomics functional profiling using HMP Unified Metabolic Analysis Network (HUMANn version 2.8.2). The details of the HUMANn2 profiling methods have been previously described [19] and were also described in the Supplementary Materials (available in the online version of this article). We used relative abundance thresholds of 0.01% for pathways present in at least 10% of samples to filter non-representative metabolic pathways.

Statistical analyses

We calculated alpha diversity using the Chao1 index based on taxonomic profiling results of the microbial species for each sample. We compared alpha diversity over time using paired Wilcoxon tests. The Bray-Curtis dissimilarity metric was calculated for each sample and was used for principal coordinate analysis to evaluate the differences in the taxonomic composition at the species level.

We performed a permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis dissimilarities (999 permutations) to quantify the percentage variance of the microbial community explained by age, time since OC initiation, and serum levels of sex hormones. We evaluated changes in the relative abundance of microbial species and metabolic pathways over time using the multi-variable linear mixed model in MaAsLin2 1.4.0 (<http://huttenhower.sph.harvard.edu/maaslin2>) [20]. All models included time since OC use and age at enrollment as fixed effects and each participant's identifier as a random effect. We also examined the distribution of serum levels of sex hormones over time and identified microbial species and metabolic pathways that were associated with serum levels of sex hormones. The endogenous levels of sex hormones were mutually adjusted using linear mixed models in MaAsLin2. All *P* values were corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR). A *q* value (FDR-corrected *P* value) <0.25 was considered statistically significant, consistent with prior microbiome studies. [21, 22]

RESULTS

The baseline characteristics of all participants are shown in Fig. 2(a). Among the ten women who started OC use at an average age of 25 years, nine took combined oestrogen and progestin pills, and one was on progestin-only pills. Community alpha diversity at baseline was the highest on average. However, there was no statistically significant change in alpha diversity over time (all paired Wilcoxon *P* values>0.10, Fig. 2b). Based on the principal coordinate analysis of the species-level Bray-Curtis dissimilarity matrix, we observed that time since initiation of OC use is not a primary driver of microbial community variability (Fig. 2c). Specifically, the percentage variance of the gut microbial community explained by individual factors ranged from 9.9% for age to 2.7% for time since initiation of OC use (Fig. 2d). When comparing the changes of microbial composition at phylum/genus level over time, we observed that on average the relative abundance of phylum *Actinobacteria* and *Firmicutes* increased after initiation of OCP. In contrast, *Bacteroidetes* decreased over time (Fig. S1).

After filtering based on minimum prevalence (>10%) and relative abundance (>0.01%), a total of 159 microbial species were included in our analyses of changes in microbial species over time. The abundance of the 159 species across all study participants was summarized in Table S1. The range of the abundances was summarized in Table S2. Overall, the ten most abundant species accounted for an average of 49.5% of community abundance, including fibre metabolizers *Eubacterium rectale* and *Faecalibacterium prausnitzii*, and *Prevotella copri*, a common gut microbe associated with non-Westernized lifestyles (Fig. 3a) [23]. Since the initiation of OC use, the relative abundances of *Clostridium asparagiforme*, *Clostridium hathewayi*, *Eubacterium eligens*, and *Clostridium symbiosum* decreased over time. However, these changes did not reach statistical significance after accounting for multiple comparisons (Fig. S2, all *q* values≥0.48). After filtering non-representative metabolic pathways, a total of 272 metabolic pathways were included. The most abundant metabolic pathways generally represented the common biochemical processes, including the biosynthesis of nucleotides and metabolism of carbohydrates (Fig. 3b). In multivariable analysis, initiation of OC use was associated with enrichments in microbial functions involved in the biosynthesis of amino acids, including L-lysine, L-threonine, and L-methionine (PWY-724, *P* value=0.001, *q* value=0.20, Fig. 3c) and L-tryptophan (TRPSYN-PWY, *P* value=0.0007, *q* value=0.20, Fig. 3c), peptidoglycan biosynthesis (PWY 6471, *P* value=0.001, *q* value=0.20, Fig. 3c), and the metabolism of coenzymes during oxidation reactions (PYRIDNUCSAL-PWY, *P* value=0.002, *q* value=0.20, Fig. 3c). Specific microbial species contributing to each enriched pathway were summarized in Table S3.

For endogenous sex hormones, as expected, we observed that the level of SHBG increased after OC use (pairwise Wilcoxon *P* values <0.05, Fig. 4), indicating compliance with OC use [24]. Endogenous levels of estradiol, progesterone, and total testosterone did not change significantly following OC initiation (Fig. 4). In longitudinal analyses of microbial composition with serum levels of sex hormones, we observed several significant associations between endogenous levels of sex hormones and the relative abundance of microbial species, as summarized in Fig. 5(a). Specifically, we observed that endogenous levels of estradiol and SHBG were positively associated with relative abundances of *Eubacterium ramulus*, a flavonoid-degrading species (SHBG: *P* value=0.0005, estradiol: *P* value=0.0007, both *q* values=0.12, Fig. 5b). Lower levels of estradiol and higher levels of total testosterone were significantly associated with more abundant *Megasphaera unclassified* (estradiol: *P* value=0.0008, *q* value=0.12; total testosterone: *P* value=0.0001, *q* value=0.11, Fig. 5b). Further, SHBG was negatively associated with *Bifidobacterium animalis*, a lactic-acid-producing anaerobe (*P* value=0.0004, *q* value=0.12, Fig. 5b). Endogenous progesterone level was positively associated with the relative abundance of *Eubacterium ventriosum* (*P* value=0.002, *q* value=0.16, Fig. 5b). When evaluating the metabolic pathways in relation to endogenous levels of measured sex hormones, we observed several suggestive associations between serum levels of sex hormones and the relative abundance of the metabolic pathways. The top ten pathways with the smallest *P* values were shown in Fig. 6(a) (all *P* values<0.003). After correction for multiple comparisons, pathways of amino acid biosynthesis (L-lysine, L-threonine, and L-methionine) remained significantly enriched in women with modest decrease in endogenous levels of estradiol (*P* value=0.0004, *q* value=0.16), lower levels of SHBG (*P* value <0.0001, *q* value=0.02) and those with higher levels of total testosterone (*P* value=0.0001, *q* value=0.10, Fig. 6b).

DISCUSSION

In a longitudinal study of ten healthy women, we demonstrated that initiation of OC use was not associated with a significant change in the gut microbiome diversity and composition. OC use was, however associated with marginal changes in the function of the gut microbiome. In addition, we observed statistically significant associations between endogenous sex hormones

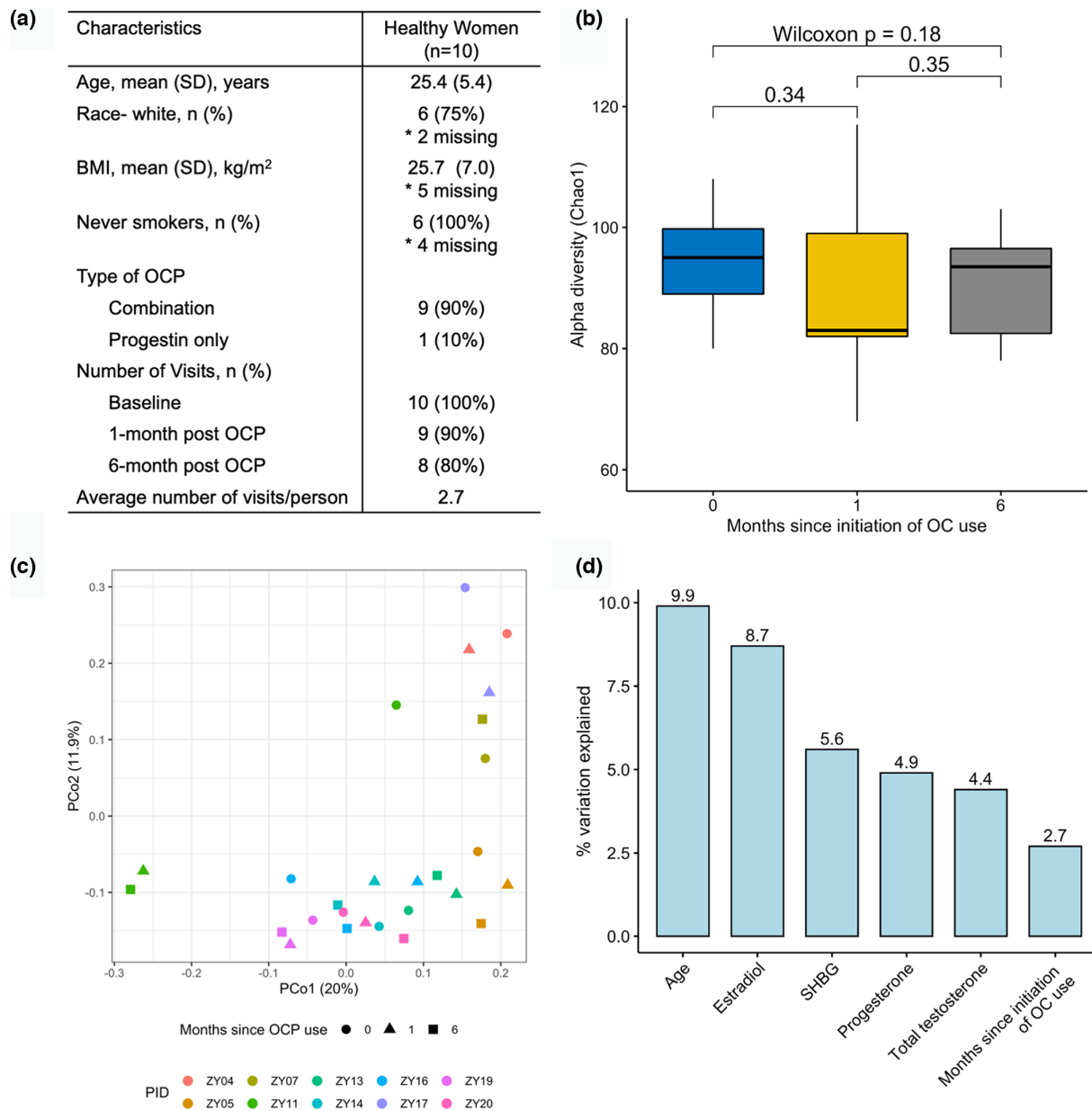


Fig. 2. Characteristics of study participants and gut microbiota. (a). Summary of participants' characteristics; (b) Alpha diversity (Chao1 index) over the study period; (c) Principal coordinates analysis (PCoA) of study participants based on Bray-Curtis distances between gut metagenomic species profiles; (d) Months since initiation of OC use account for 2.7% of variance whereas estradiol accounts nearly 9% of the variance based on permutational multivariate analysis of variance (PERMANOVA) of the Bray-Curtis dissimilarity matrix. BMI: body mass index; OC: oral contraceptive; SD: standard deviation; SHBG: sex hormone binding globulin.

and the composition and function of the gut microbiome. Specifically, the relative abundance of *Eubacterium ramulus* was positively associated with changes in serum estradiol and SHBG. In addition, the metabolic pathway of L-lysine, L-threonine, and L-methionine biosynthesis was significantly enriched after the initiation of OC use. The enrichment was also significantly associated with a modest decrease in endogenous levels of estradiol and SHBG and an increase in total testosterone.

Previous studies support our observation that OC use is not associated with compositional changes of gut microbial species over time. A population-based metagenomics analysis reported cross-sectional OC use only explained 0.2% of the interindividual variation in microbial composition based on the Bray-Curtis distance [25]. The significant difference in the distribution of age (Mean=44.8, SD=13.4 years) and comorbidities in this study compared to our study population likely accounted for the lower contribution of OC use to the interindividual variation of gut microbial composition. In support of this, a recent

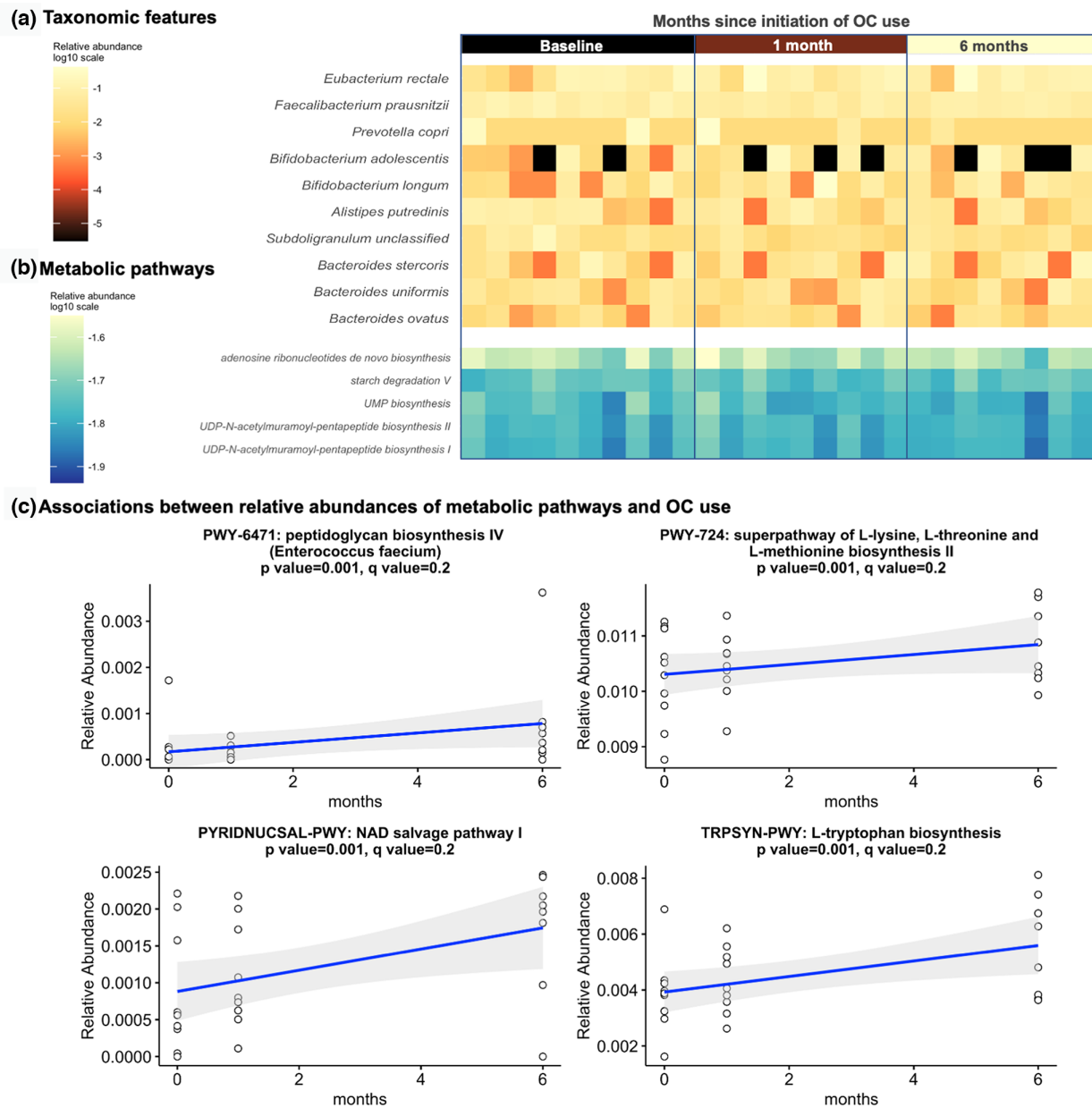


Fig. 3. Taxonomic and functional profiles of the gut microbiome over time. (a) Distribution of the ten microbial species most abundant on average; (b) Distribution of the five most metagenomically abundant pathways relative abundance of the metabolic pathways significantly associated with time since initiation of OC use (q values < 0.25). (c) Scatter plots of the metabolic pathways significantly associated with time since initiation of OC use (all q values = 0.20). All q values were derived from the multivariable-adjusted models and corrected for multiple comparison using the Benjamini-Hochberg false discovery rate method.

cross-sectional study of healthy women showed that reported OC use explained 4% of the interindividual variation of the gut microbial composition [26], consistent with our finding. Additionally, a prospective study of 17 overweight/obese women with polycystic ovary syndrome showed no significant changes in alpha diversity following 3 month use of OC [27]. Nevertheless, our study that leveraged metagenomic sequencing and enrolled new OC initiators who were followed for 6 months significantly expands on prior studies and provides a more in-depth and generalizable characterization of the gut microbiome.

We found enrichment of microbial function for the biosynthesis of peptidoglycan, the main component of bacteria cell walls (PWY 6471), with the initiation of OC. This pathway is particularly enriched in Gram-positive Enterococci, including *Enterococcus faecium* and *Enterococcus hirae*. This finding is consistent with a prior large cross-sectional study by Jackson and colleagues that showed OC users had a higher abundance of *Enterococcaceae*, a family that includes the lactic-acid-producing genus *Enterococcus* [28].

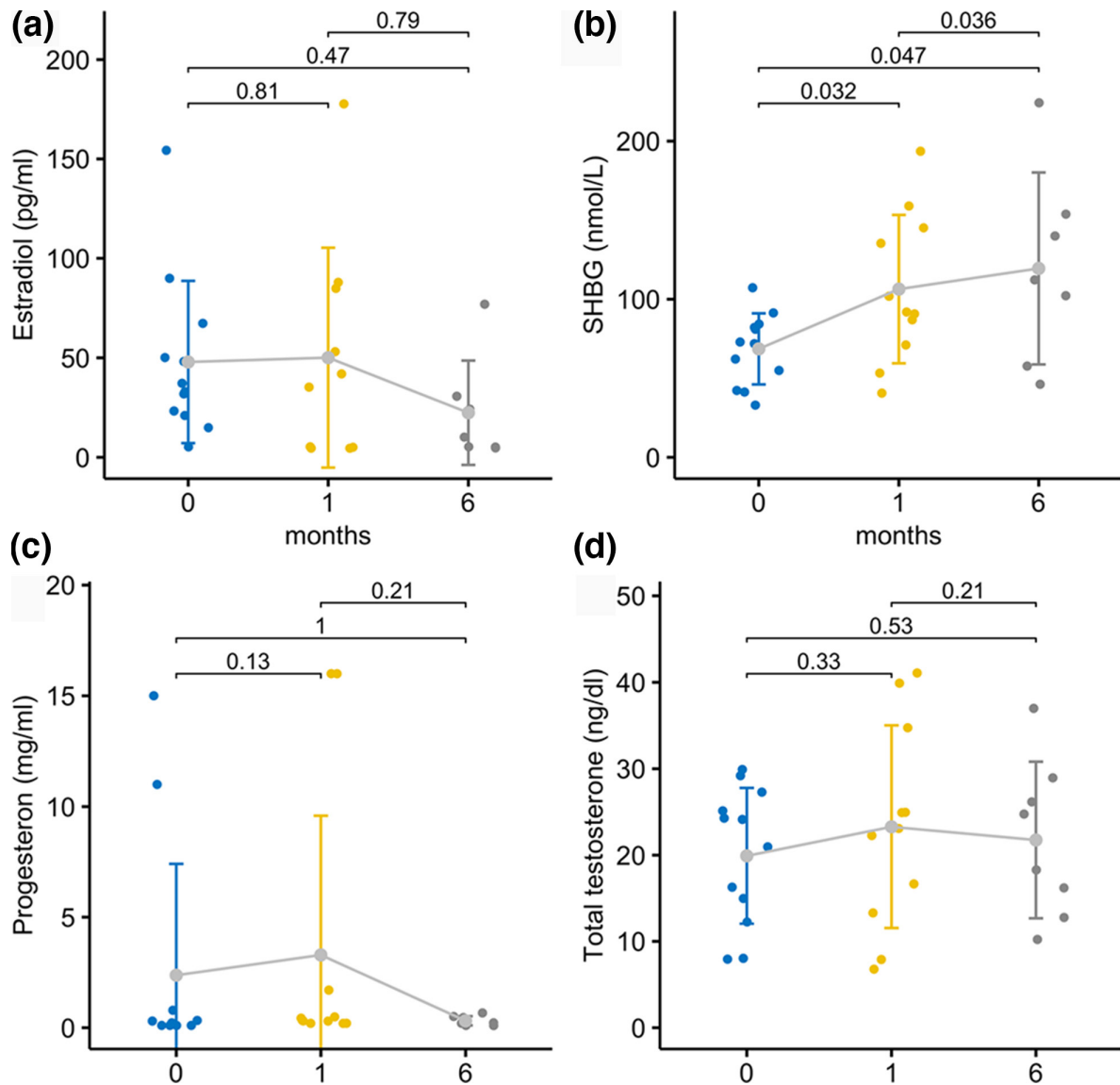


Fig. 4. Serum level of sex hormones over time. The mean and standard error of each marker were plotted over time from baseline, 1 month, to 6 months since initiation of OC use for (a) estradiol, (b) sex hormone binding globulin (SHBG), (c) progesterone, and (d) total testosterone.

Our data also showed that endogenous levels of estradiol and SHBG were positively associated with the relative abundance of *Eubacterium ramulus*, a flavonoid-degrading anaerobe commonly involved in fermentation processes in the human gastrointestinal tract [29, 30]. Further, we also observed an enrichment of microbial functions of mixed acid fermentation associated with elevated levels of total testosterone. Although human studies evaluating direct associations between endogenous levels of sex hormones and the composition of the gut microbiome are scarce, animal studies have suggested an important role for estradiol in shaping the gut microbiota. For example, Kaliannan *et al.* [31] reported that female mice undergoing ovariectomy experienced a significant shift in gut microbiome composition, with beta diversity (Bray-Curtis dissimilarity matrix) more similar to male mice than normal female mice. The same study further demonstrated treatment with estradiol and estrogen-like compounds (e.g. isoflavones) can induce substantial changes in the gut microbiome, including decreased *Proteobacteria* and lipopolysaccharide biosynthesis and can protect against metabolic syndrome in male and ovariectomized mice [31]. These findings have in part been corroborated by other groups that have demonstrated that subcutaneously implanted estradiol treatment has a significant impact on the gut microbiome of male and female mice [32, 33]. Further, Shin *et al.* [14] found that serum estradiol was associated with the relative abundance of several gut microbial genera, including *Slackia*, *Lactococcus*, and *Butyrivibrio* (butyrate-producing bacteria). Together, the evidence from both animal and human studies supports our

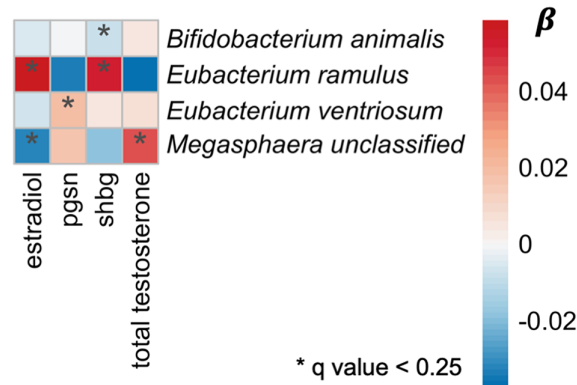
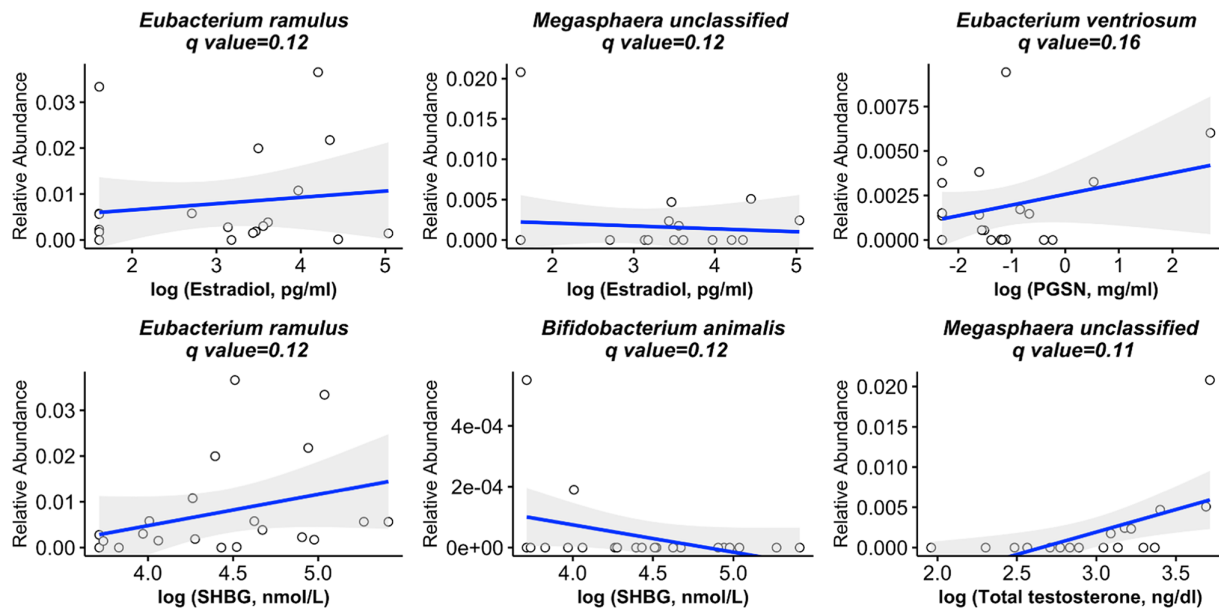
(a) Microbial species and serum sex hormones**(b) Microbial species significantly associated with sex hormones**

Fig. 5. Microbial species and endogenous sex hormones. (a) Associations between serum levels of sex hormones and the relative abundance of microbial species. The endogenous levels of sex hormones included estradiol, progesterone (pgsn), sex hormone binding globulin (shbg), and total testosterone. (b) Relative abundances of microbial species significantly associated with natural log transformed serum sex hormone levels (q values < 0.25). All serum sex hormones were mutually adjusted in addition to age at enrollment and time since OC initiation in multivariable linear mixed models using MaAsLin2. All q values were derived from the multivariable-adjusted models and corrected for multiple comparison using the Benjamini-Hochberg false discovery rate (FDR) method.

observation that serum estradiol is associated with gut microbiome composition. It also suggests a possible link between serum estradiol and gut microbes involved in flavonoid degradation pathways.

The limitation of our study includes the modest sample size, which did not allow us to adjust for additional potential confounders including lifestyle and dietary factors. It also limited our statistical power to identify more modest compositional and functional changes. In addition, we weren't able to separately evaluate the type of OC used in our study or assess OC use among individuals with underlying immune-mediated conditions who may respond differently to these medications. However, our study also has several strengths. The longitudinal study design with repeated measurements allowed us to evaluate gut microbiome changes after the initiation of OC use. It minimized confounding due to inter-individual differences in environmental factors that may modify the composition of the gut microbiome. In addition, the serum samples were all collected at the follicular stage from each participant to minimize the influence of normal physiologic changes on sex hormone measurements.

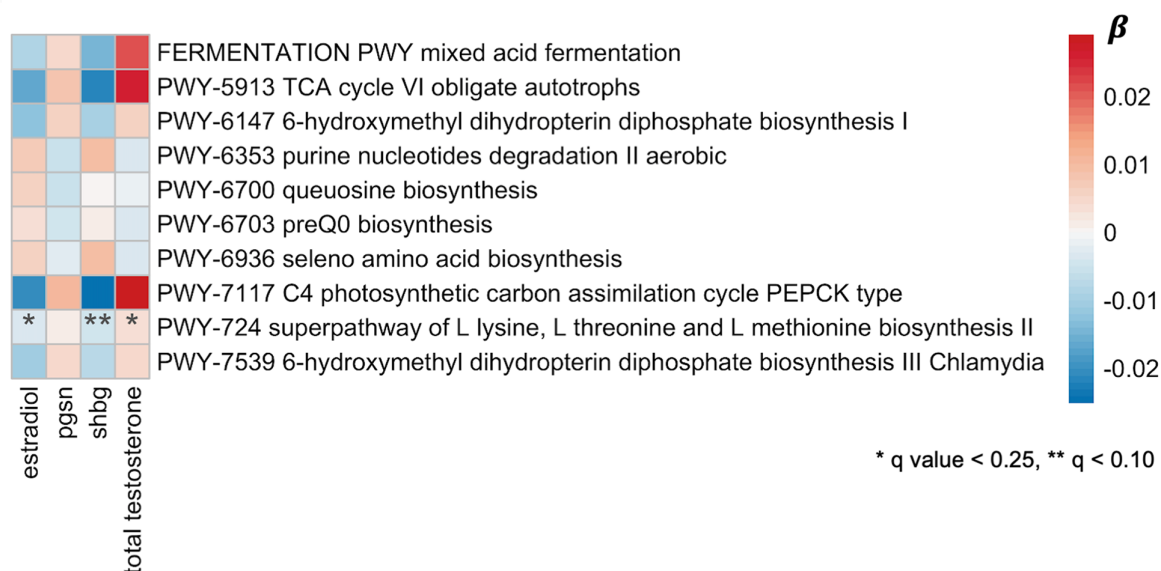
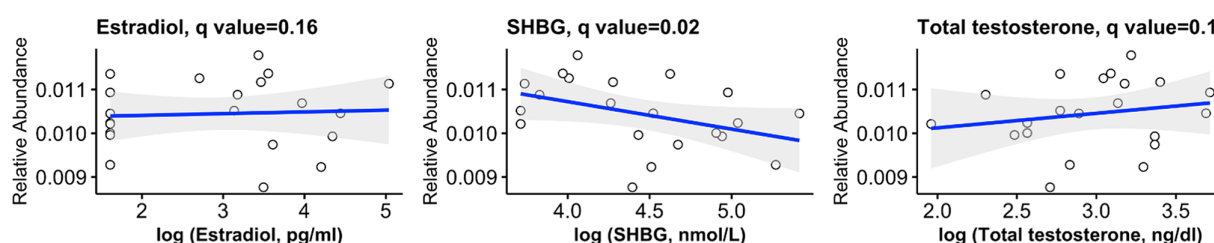
(a) Metabolic pathways and serum sex hormones**(b) PWY-724 : superpathway of L-lysine, L-threonine and L-methionine biosynthesis II significantly associated with sex hormones**

Fig. 6. Metabolic pathways and serum sex hormones. (a) Associations between serum sex hormones and the relative abundances of top ten metabolic pathways (P values < 0.003); (b) PWY-724: superpathway of L-lysine, L-threonine and L-methionine biosynthesis II was significantly associated with estradiol, sex hormone binding globulin (SHBG), and total testosterone (q values < 0.25). All serum sex hormones were mutually adjusted in addition to age at enrollment and time since OC initiation in multivariable linear mixed models using MaAsLin2. All q values were derived from the multivariable-adjusted models and corrected for multiple comparison using the Benjamini-Hochberg false discovery rate (FDR) method.

CONCLUSIONS

Our study shows that OC initiation is not associated with significant changes in the community structure of the gut microbiome in healthy women. In contrast, endogenous sex hormones appear to be more closely linked with the composition and function of the gut microbiome, particularly with flavonoid-degrading species, such as *E. ramulus*. Future large-scale studies are needed to validate our study findings and better characterize the associations between OC use, endogenous sex hormone, and changes in the gut microbiome, particularly among individuals with immune-mediated diseases.

Data availability

The metagenomic sequencing data have been deposited at Sequence Read Archive under BioProject accession: PRJNA750475. It can be accessed at: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA750475>. In addition, the individual-level metadata that supports the findings of this study is available upon reasonable request from the corresponding author Dr Hamed Khalili.

Funding information

This research is supported by H. Khalili's career development award from the National Institute of Diabetes and Digestive and Kidney Diseases (K23 DK099681).

Acknowledgements

The authors thank all study participants and staff at Mayo Clinic and MGH NextGen Sequencing Core.

Author contributions

X.H., participated in the data analysis, interpretation of the data, drafting, and critical revision of the manuscript; Y.C., participated in the acquisition of data, drafting, and critical revision of the manuscript; D.M.M., K.M., S.M.C., D.B., participated in the acquisition of data and critical revision of the manuscript; H.K., participated in the study design, acquisition and interpretation of data, and critical revision of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

H.K., has received consulting fees from Takeda and research funding from Takeda and Pfizer.

Ethical statement

This study was approved by the Institutional Review Board at Mass General Brigham (Protocol # 2013P002125). Informed consent was obtained from all participants. We obtained consent for publications from all study participants and co-authors.

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