Combined hormonal contraceptives are associated with minor changes in composition and diversity in gut microbiota of healthy women

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Summary

Recent human and animal studies have found associations between gut microbiota composition and serum levels of sex hormones, indicating that they could be an important factor in shaping the microbiota. However, little is known about the effect of regular hormonal fluctuations over the menstrual cycle or CHC-related changes of hormone levels on gut microbiota structure, diversity and dynamics. The aim of this study was to investigate the effect of CHCs on human gut microbiota composition. The effect of CHC pill intake on gut microbiota composition was studied in a group of seven healthy premenopausal women using the CHC pill, compared to the control group of nine age-matched healthy women that have not used hormonal contraceptives in the 6 months prior to the start of the study. By analysing the gut microbiota composition in both groups during one menstrual cycle, we found that CHC usage is associated with a minor decrease in gut microbiota diversity and differences in the abundance of several bacterial taxa. These results call for further investigation of the mechanisms underlying hormonal and hormonal contraceptive-related changes of the gut microbiota and the potential implications of these changes for women’s health.

Introduction

Oral combined hormonal contraceptives (CHCs) are among the most frequently used methods of birth control and are used by an estimated 16% (151 million) of women of reproductive age (15–49 years old) (United Nations 2019). CHCs consist of natural or synthetic versions of 17β-estradiol (hereafter referred to as estradiol) and progesterone that, by a negative-feedback loop mechanism, inhibit secretion of the two pituitary hormones that govern the menstrual cycle: follicle stimulating hormone and luteinizing hormone. In the absence of these two pituitary hormones, there is no induction of estradiol and progesterone synthesis. Without sufficient circulating estradiol, ovulation does not occur and pregnancy is prevented (Kumar and Sait, 2011; Christin-Maitre, 2013). The decrease of serum estradiol and progesterone levels due to oral CHC application (Christin-Maitre, 2013; Roos et al., 2015) has been correlated to various physiological side-effects such as migraines (Calhoun and Batur, 2017), risk of cardiovascular diseases (dos Santos et al., 2014; Lima et al., 2017), weight gain (Marnach et al., 2013; Cipriani et al., 2019) and decreased bone density (Grandi et al., 2016). Although the gut microbiota has recently been shown to have profound effects on gut/systemic health and widespread effects on endocrine function (Patterson et al., 2016; Valdes et al., 2018; Gribble and Reimann, 2019), there is currently no information on the effect of CHC-induced hormonal changes on gut microbiota structure and function. The gut microbiota plays a significant role in the regulation of host’s energy metabolism by affecting the secretion of insulin and lipid metabolism (Bäckhed et al., 2005; Turnbaugh et al., 2006; Saad et al., 2016; Li et al., 2017).
secretion of cortisol (Wang and Wang, 2016) and gastrointestinal peptide hormones that regulate satiety and energy expenditure (Lupien-Meilleur et al., 2020). Microbiota-derived tryptophan is a serotonin precursor that regulates gut motility and may affect behaviour (Agus et al., 2018). Additionally, gut microbiota changes have been related to thyroid dysfunction (Knezicevic et al., 2020), although the underlying mechanisms are not yet clear. Recently, studies have shown a link between gut microbiota and sex steroid hormones. A study from 2018 showed a significant difference in gut microbiota composition between male and female mice. Furthermore, the same study reported a change in gut microbiota composition in mice that underwent ovariectomy (Kaliannan et al., 2018), and other studies have demonstrated that estradiol treatment of male and female mice results in shifts in the gut microbiota (Benedek et al., 2017; Acharya et al., 2019). Consistent with preclinical studies, human studies have also reported associations between systemic estradiol levels and gut microbiota composition. Differences in gut microbiota composition and richness between men and women have been found in several studies, suggesting that sex hormones may play a role in gut microbiota homeostasis and development (Mueller et al., 2006; Singh and Manning, 2016; Gao et al., 2018; Sinha et al., 2019). One indication that estradiol can affect microbiota composition comes from the study of menopausal women, who have significantly decreased systemic estradiol levels. Compared to premenopausal women, gut microbiota of women in menopause had lower alpha diversity (Flores et al., 2012; Fuhrman et al., 2014). Also, recent work by Shin et al. (2019) found that gut microbiota diversity was positively correlated to serum testosterone and estradiol levels in adults. In spite of ample evidence demonstrating a link between steroid sex hormones and the gut microbiota, the potential effects of CHCs-induced hormonal changes on gut microbiota structure and function have not been addressed so far. As the gut microbiota affects many aspects of host health and the endocrine system, potential CHCs-related changes in gut microbiota composition may provide an explanation for physiological changes associated with CHCs application and shed light on thus far unconsidered CHCs-related health risks. This calls for the investigation of CHCs-associated gut microbiota.

Since CHCs function on the principle of maintaining low levels of circulating estradiol and progesterone, we hypothesized that CHCs would affect gut microbiota composition and diversity. As previous studies in animals and human trials showed that low estradiol and progesterone levels are associated with decreased microbiota diversity, we were expecting to observe a similar trend and aimed at identifying the most affected. In order to assess the effects of CHCs on gut microbiota, we compared the gut bacterial community composition of a limited cohort of healthy pre-menopausal women using CHCs (n = 7) and age-matched women not using CHCs (n = 9). By analysing 16S rRNA gene amplicon sequencing data from stool samples collected every other day over the 4-week menstrual cycle (202 samples in total), we found that CHC usage is associated with a significantly lower gut microbiota diversity. Furthermore, we identified bacterial genera that are differentially abundant in the control and the CHC groups. Our results suggest that CHC-induced hormonal changes may affect gut microbiota diversity and call for further investigation of the mechanisms underlying these alterations and their potential implications for women’s health.

Results

Study design

The present study has been approved by the local Ethic’s commission of the Medical University of Vienna (EK-Nr.: 2112–2017). All participants gave written informed consent before participating in the study. The aim of the present study was to assess the effect of CHC-induced changes in hormonal fluctuations on gut microbiota composition and diversity. To this end, we compared the gut microbiota of volunteers using CHCs (n = 7; Details on CHCs in Supplementary Table 1) with a control group of age-matched participants (n = 9). All participants were healthy pre-menopausal women between the ages of 21 and 29 (Supplementary Table 2). The body mass index (BMI) of the participants ranged from 18.84 to 27.74 and there was a trend for a lower BMI in the CHC group (mean CHC: 20.75, mean control: 23.26, Student’s t-test, p = 0.054). The participants were advised to follow a Mediterranean diet (Supplementary Table 3) and to report average daily intake of carbohydrates, protein and fat (Supplementary Fig. 1) for 2 weeks prior to, as well as during the period of stool sampling (4 weeks). There were no significant differences (Wilcoxon rank-sum test with continuity correction) in average intake of carbohydrates (p = 1), protein (p = 0.11) nor fat (p = 0.67) between the control and CHC group. Participants began collecting stool samples on the first day of their menstrual cycle and continued with sample collection approximately every other day for the full length of the cycle (28 days; Supplementary Tables 4 and 5). In parallel, blood samples were taken weekly in order to monitor the serum hormonal fluctuations (Supplementary Table 6).

CHCs alter serum levels of estradiol and progesterone

We analysed the serum levels of the two hormones most directly affected by CHC intake: estradiol and progesterone.
progesterone (Supplementary Table 6). In the control group, we observed the expected two major phases of the menstrual cycle, with relatively low and slowly increasing estradiol and progesterone levels during the first 15 days of the cycle (follicular phase), peaking at around day 15 and remaining elevated from day 15 to day 28 (luteal phase) (Supplementary Fig. 2A). By contrast, due to intake of the CHCs, serum estradiol and progesterone in the CHC group do not show any significant fluctuation (Supplementary Fig. 2A) and remain at significantly lower concentrations compared to the control group (Supplementary Fig. 2B).

Microbiota beta diversity is not affected by CHC intake

We evaluated the gut microbiota composition in the cohort by sequencing the V3–V4 region of bacterial 16S rRNA genes. A total of 202 samples (114 in control group and 88 in CHC group) were collected and sequenced. We recovered 222 amplicon sequencing variants (ASVs) that constituted at least 1% of reads in at least three samples of the complete dataset (Additional File 1). In order to assess if CHC use was associated with changes in gut microbiota structure, we analysed beta diversity between the gut bacterial community of the control and the CHC group.

Ordination of beta diversity did not show clustering of samples by group (Fig. 1A), though samples from participants 3 and 4 (CHC group) did cluster separately from all other samples (Fig. 1A). Consistent with this, non-parametric multivariate analysis of variance revealed that CHC use did not have a statistically significant effect on beta diversity (PerMANOVA: \( r^2 = 0.04, p = 1 \)). Since we had observed a significant increase of serum estradiol and progesterone in the luteal phase of the cycle in the control group, we wanted to examine if this hormonal change from low (follicular phase) to high levels (luteal phase) would be reflected in beta diversity. Beta diversity was not significantly different between the follicular and luteal phase (Fig. 1B; PerMANOVA: \( r^2 = 0.008, p = 0.75 \)), suggesting that serum estradiol and progesterone are not a major factor in shaping the gut microbiota beta diversity.

Alpha diversity is associated with CHC intake

We sought to further assess the gut microbiota structure in correlation with the CHC intake-induced hormonal changes by analysing alpha diversity in both groups of samples. We found that the alpha diversity, as measured by the number of observed sequence variants (ASVs), Shannon diversity index and Simpson diversity index, was significantly decreased in the CHC compared to the control group (Fig. 2A). Since we had observed a significant difference in estradiol and progesterone serum levels between the two groups (Supplementary Fig. 1B), we hypothesized that hormonal shifts could be a contributing factor for the recorded decrease in alpha diversity. Therefore, we evaluated if the shift from low (follicular) to high (luteal) estradiol/progesterone phase coincides with a change in alpha diversity in the control group. However, alpha diversity in the control group was not significantly different between follicular and luteal phase of the cycle (Fig. 2B).

CHC intake is associated with changes in the relative abundance of major bacterial genera

To further explore the diversity changes in relation to the CHC intake that might be driving the observed changes in alpha diversity, we identified taxa whose relative
abundance was significantly different in CHC samples compared to controls. In order to limit the effect of individual participant variation and to focus on the genera that are common yet differently abundant in both groups, we considered only genera that were found in at least four participants (44% of the group) in the control and at least three participants (42% of the group) in the CHC group. We found five genera that showed a minimum twofold change in abundance between the control and CHC group (Fig. 3A; Table 1). Three of those genera: *Eubacterium*, *Haemophilus* and *unclassified Firmicutes* were less abundant in the CHC group, while *Akkermansia* and *Barnesiella* were enriched in the CHC group (Fig. 3A; Table 1, Supplementary Table 7). Since we hypothesized that the changes in the gut microbiome composition in the CHC group compared to the control group might be driven by a decrease in estradiol and progesterone levels, we examined which genera were differentially abundant between the high (luteal) and low (follicular) estradiol/progesterone cycle phase in the control group. Interestingly, this revealed that the genera *Akkermansia* and *Lactococcus* were significantly more abundant (≥4× and 2× enriched respectively) during the high estradiol/progesterone (luteal) phase compared to the follicular phase in the control group (Fig. 3B; Table 1, Supplementary Table 8).

**Discussion**

In the present study, we evaluated whether CHC usage was associated with changes in gut microbiota composition of healthy women. Although studies in mice have suggested that estradiol plays a role in shaping gut microbiota composition (Kalianan et al., 2018; Acharya et al., 2019), we did not find significant differences in beta diversity between the control and the CHC group. Similarly, several studies in humans have not found a difference in beta diversity between men and women (Mueller et al., 2006; Ding and Schloss, 2014; Dominian et al., 2015; Singh and Manning, 2016; Gao et al., 2018; Takagi et al., 2019) or pre- and post-menopausal women (Flores et al., 2012; Fuhrman et al., 2014). Precise evaluation of the impact of estradiol on the human gut microbiota is challenging due to the multitude of potentially confounding factors, such as diet (Gentile and Weir, 2018), exercise (Clark and Mach, 2016) and other endocrine factors (Sandhu et al., 2017) that affect the gut microbiota composition. However, some studies have shown a decrease in gut microbiota richness related to lower estradiol and progesterone levels in menopausal women (Flores et al., 2012; Fuhrman et al., 2014; Goedert et al., 2015). Consistent with these results, we found that the CHC group had significantly lower gut microbiota richness, which may be a consequence of the
CHC-induced decrease in estradiol and progesterone. Individual effects of estradiol and progesterone on gut microbiota composition are difficult to discern in our study, since CHCs significantly reduce the circulating amount of both hormones. Although studies on ovariectomized mice that involved estradiol or progesterone treatments showed that both hormones induce changes in gut microbiota composition (Kaliannan et al., 2018; Nuriel-Ohayon et al., 2019; Sovijit et al., 2019) such distinctive analyses are significantly more challenging to perform in human studies, especially in cycling women. Most human microbiota studies that report menopause, gender or pregnancy-related shifts in gut microbiota cannot precisely distinguish between estradiol and progesterone effects, because their surges and decreases largely overlap.

Interestingly, even though CHCs-related low estradiol and progesterone coincided with a decrease in gut microbiota diversity, we could not confirm that short-term estradiol and progesterone shifts occurring during regular

Fig. 3. Differentially abundant genera. DESeq2: Genera with differential abundance (Log10 of abundance percentage) in the CHC compared to the control group (A) and in the luteal compared to the follicular phase of the menstrual cycle in the control group (B). [Color figure can be viewed at wileyonlinelibrary.com]
menstrual cycle (follicular to luteal phase switch) result in significant changes in gut microbiota composition or richness. This is in contrast to the studies of human vaginal microbiota, where estradiol plays a significant role in community change between low and high estradiol phase of the cycle. Namely, a main *Lactobacillus*-dominated vaginal microbiota undergoes a change during menses (lowest estradiol point of the cycle), marked by a decrease in *Lactobacillus* abundance and increase in diversity. This shift is, however, transitional and the *Lactobacillus*-dominated community is restored with estradiol surge in late follicular and luteal phase (Gajer et al., 2012; Song et al., 2020). A more complete understanding of gut microbiota changes in relation to menstrual cycle hormonal fluctuations requires further investigation.

The observed decrease in alpha diversity in the CHC group could be explained by a significantly lower abundance of unclassified *Firmicutes*, *Eubacterium* and *Haemophilus* in the CHC group. Relative abundance of *Eubacterium* is particularly low in the CHC group, where this taxon was not detected in two participants (3 and 16) and relative abundance in other participants is lower than 1% (Supplementary Table 7). The mechanism by which CHCs may cause changes in abundances of the above-noted genera is not clear, and most of them have not been correlated to changes in sex hormones according to the current literature. The expansion of *Haemophilus* in the control group of our study is not in any obvious relation to estradiol and progesterone changes. Presence of *Haemophilus* species has been correlated with gut microbiomes of individuals who have chronic diseases and altered immunity such as multiple sclerosis, tuberculosis and hepatitis B (Chen et al., 2016; Wang et al., 2017; Hu et al., 2019). In our study, none of the participants was diagnosed with a chronic disease which could explain the increased abundance of *Haemophilus*, which leaves a possibility that the expansion of *Haemophilus* in the control group could be due to higher estradiol or progesterone levels. CHC-induced lower estradiol levels might be the cause of *Akkermansia* increase in the CHC group. Consistent with our results, studies on female mice have found a decrease in abundance of *Akkermansia* upon treatment with estradiol or estrogen compounds such as bazedoxifene or phytoestrogens (Chen et al., 2018; Hamm et al., 2019), implying a negative correlation between *Akkermansia* abundance and oestrogen or estrogenic compounds.

Analyses of gut microbiota composition between follicular and luteal phase in the control group showed differential abundances in genera *Lactococcus* and *Akkermansia*. Enrichment of *Lactococcus* in the luteal phase in the control group may be explained by the increase in estradiol, which is in line with a study in ovariectomized mice that reported an increase in *Lactococcus* abundance upon estradiol treatment (Paul et al., 2017). Surprisingly, and contrary to previously observed association of *Akkermansia* with low estradiol/progesterone in the CHC group, we found that *Akkermansia* was enriched in the high estradiol/progesterone (luteal) phase of the menstrual cycle in the control group. A similar effect was shown in a mouse study where treatment with bisphenol A, an estrogenic metabolic disruptor, resulted in higher abundance of *Akkermansia* in gut microbiota of male mice (Javurek et al., 2016). This implies a rather complex role of estradiol and estrogenic compounds in the growth of *Akkermansia*, which requires further analysis. In our study, the CHC-group had constantly low estradiol and progesterone levels that did not reach hormone levels in the control group. Therefore, it may be hypothesized that *Akkermansia* is sensitive to the levels of circulating estradiol and/or progesterone, which in the control group never get as low as in the CHC group. It is also possible to imagine that there may be differences between different *Akkermansia* strains in relation to estradiol/progesterone-regulated growth, which would be interesting to examine in wider population. Indeed, 16S rRNA gene analyses of human gut microbiota indicated that there may be eight *Akkermansia*-like species. Although comparative metagenomic analyses show that this bacterium is a specialized mucin degrader, there are unexplored differences between genomes of *Akkermansia*-like species that could potentially be involved in response to hormonal

Table 1. Taxa with a significant change in abundance.

| Genus           | Fold change | p-adjusted | Comparison          |
|-----------------|-------------|------------|---------------------|
| *Akkermansia*   | 5.274374    | 1.14e-02   | CHC to control      |
| *Barnesiella*   | 2.167452    | 7.31e-09   | CHC to control      |
| *Eubacterium*   | −7.225012   | 3.96e-15   | CHC to control      |
| *Haemophilus*   | −2.143547   | 0.0006     | CHC to control      |
| *unclassified Firmicutes* | −2.718856 | 3.79e-07  | CHC to control      |
| *Akkermansia*   | 5.205367    | 3.17e-09   | Follicular to luteal|
| *Lactococcus*   | 2.158456    | 0.0071     | Follicular to luteal|

Taxa with a significant change in abundance in CHC compared to the control group, as well as in follicular compared to the luteal phase according to the DESeq2 algorithm.
fluctuations (Geerlings et al., 2018). As our sequencing data do not allow accurate taxa differentiation below genus level, we cannot determine whether the observed differences in Akkermansia abundances are due to presence of different species between groups. Therefore, further functional and in vitro studies are warranted to examine the mechanisms by which steroid sex hormones affect Akkermansia’s growth.

In the present study, we show that CHC-related hormonal changes cause a minor decrease in gut microbiota alpha diversity and alter abundances of several bacterial genera. Even though there are certain limitations in our study, our results suggest that CHSs application may be associated with insofar unconsidered changes in gut microbiota that call for further investigations. Like many gut microbiota studies in healthy individuals, our study had a limited cohort of 16 participants and larger cohort studies are needed to confirm and further explore our findings. Also, our study did not include cross-over analyses of gut microbiota in participants before and after CHCs application, which would allow closer examination of gut microbiota changes and potential recovery after CHCs application. As outlined in the Discussion, due to overlapping estradiol and progesterone peaks, it is difficult to analyse the individual effects of these hormones. Similar to standard observational studies of gut microbiota in healthy individuals, our study design could not explain whether estradiol and progesterone-induced gut microbiota changes by directly interacting with gut bacteria or indirectly, by affecting other physiological processes that regulate gut homeostasis. This calls for further investigations of the mechanisms by which estradiol and progesterone affect the gut microbiota composition in controlled animal models. In spite of these limitations, we believe that our results will prompt future investigations in larger healthy cohorts to confirm and better understand the sex steroid hormone-related gut microbiota changes. Additionally, in the light of CHCs-associated gut microbiota changes, future studies should examine the potential consequences of CHCs-induced gut microbiome alterations on diverse aspects of host physiology such as energy metabolism, as well as metabolism of phytosterogens, steroid hormones and steroid-like hormone signalling disruptors.

**Experimental procedures**

**Participants exclusion criteria**

All participants are women aged between 21 and 29 that had not used antibiotics in the 3 months prior to the beginning of the study. Other exclusion criteria for participation in the study were acute or chronic infectious diseases, gastrointestinal disease in the 3 months prior to entering the study, polycystic ovary syndrome, oligomenorrhea, anovulation or other disturbances of the menstrual cycle. In the CHC group, the participants had been using combined oral contraceptives containing a form of oestrogen and progestin (Supplementary Table 1). Inclusion criterion for all participants was eumenorrhea, average length of cycle was 28.75 days in the control group and 28.16 days in the CHC group. In the control group participants reported not having used oral hormonal contraceptives 6 months prior to the beginning of the study.

**Dietary recommendations during the study**

All participants followed a Mediterranean diet. The dietary intervention was based on the PREDIMED recommendations http://www.predi.med.es/pyramids.html. 8 weeks prior to study start patients attended an individual dietary training session on the Mediterranean diet. According to the Mediterranean diet pyramid, diet recommendations are summarized in Supplementary Table 2. The goal for ideal macronutrients distribution was set to 35%–40% fat, 15%–20% protein and 40%–50% carbohydrates (Estruch et al., 2018). During the study period, participants recorded a food diary. The food diary and therefore composition of the macronutrients was analysed with Nut.s Science https://www.nutritional-software.at/content/nuts-software/nuts-science/. No total calorie restriction was advised nor was physical activity promoted.

**Stool sample collection**

Participants in both groups collected stool samples from the first day of menses and continued sampling approximately every other day during a 28-day menstrual cycle (Supplementary Tables 4 and 5). Samples were immediately stored at −20°C until they were used for DNA extraction.

**Blood sample collection and serum hormone measurement**

Blood samples were taken after an overnight fasting period of >10 h. In line with the international standards of laboratory methods, the analysis was conducted in the certified Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna (http://www.kimcl.at/).

**DNA extraction and 16S rRNA gene amplicon sequencing**

DNA from stool samples was extracted using the QiAamp DNA Stool Mini Kit (Qiagen) following the manufacturer’s instructions. Before extraction, samples were

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subjected to mechanical lysis in ASL Buffer, by bead beating the samples two times on the FastPrep-96 Instrument (MP Biomedicals) for 45 s at 1400 rpm. The V3–V4 region of the bacterial 16S rRNA gene was amplified (30 cycles) from the DNA using the 341F and 785R primers (Klindworth et al., 2013) which were modified with a linker sequence (Herbold et al., 2015) and barcoded (8 cycles) in a unique dual setup. Thereafter, barcoded samples were purified and normalized over a SequaPrep™ Normalization Plate Kit (Invitrogen) using a Biomek® NXP Span-8 pipetting robot (Beckman Coulter) and pooled and concentrated on columns (Anlaytik Jena). Indexed sequencing libraries were prepared with the Illumina TruSeq Nano Kit as described previously (Herbold et al., 2015) and sequenced in paired-end mode (2 × 300 nucleotide; v3 chemistry) on an Illumina MiSeq following the manufacturer’s instructions. The workflow systematically included four negative controls (PCR blanks, i.e., PCR-grade water as template) for every 90 samples sequenced. Amplicon pools were extracted from the raw sequencing data using the FASTQ workflow in BaseSpace (Illumina) with default parameters.

16S rRNA gene amplicon analysis

After sequencing, sample demultiplexing was performed with the python package demultiplex (Laros JFJ, github.com/jfjaros/demultiplex) allowing one mismatch for barcodes and two mismatches for linkers and primers. ASVs were inferred using the DADA2 R package (https://github.com/benjjneb/dada2) applying the recommended work (Callahan et al., 2016). FASTQ reads 1 and 2 were trimmed at 230 nucleotides with allowed expected errors adjusted per sequencing run. ASV sequences were subsequently classified using SINA version 1.2.11 and the SILVA database SSU Ref NR 99 release 132 (Pruesse et al., 2012) using default parameters. ASV sequences were subsequently classified using the RDP Classifier version 2.0.2 (Wang et al., 2007) with a confidence cutoff of 0.5.

Gut microbiota diversity and statistical analysis

All reads were rarefied to the minimal library size and only the ASVs that appeared in three or more samples with minimum relative abundance of 1% were further analysed. The reads table with the filtered ASVs was normalized to the minimum library size. Two ASVs (ASV_38z_b67 and ASV_r4l_7g9) identified as Aestuarissipra, an environmental genus found in aquatic samples (Park et al., 2014), were excluded from further analysis as contaminants. The resulting 222 ASVs (Additional File 1) were used for all performed analysis of diversity and taxa abundance. Analyses of alpha and beta diversity were done in R version 3.6.2 using the vegan package version 2.5.6. Differences in alpha diversity were statistically tested using the Wilcoxon rank-sum test with continuity correction from the basic R Stats package. Differences in beta diversity were tested using the adonis function from the vegan 2.5.6 package. The DESeq2 R package version 1.26.0 was used for the identification of the taxa with significantly different abundance between the CHC and the control group. ASV counts were agglomerated to genus rank and differential abundance of genera was analysed with DESeq2. Average age and BMI differences between the control and the CHC group were tested using the student’s t-test. The results were considered statistically significant if the p-value was less than 0.05.

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Authors’ Contribution

J.M. and M.L. contributed to study design, performed acquisition, analysis and interpretation of data and wrote the manuscript. B.H. contributed to data acquisition and analysis. G.K., J.S., H.R., N.S., K.M., M.B. contributed to sample acquisition and analysis. P.W. was involved in care and recruitment of the study participants. A.K.-W. and D.B. performed the design and coordination of the study, revised the data analysis and the manuscript. All the authors have read and approved the final manuscript.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1.** Supplementary Information

**Supplementary Fig. 1.** Reported dietary intake. Reported dietary intake of major nutrient types (carbohydrates, fat and protein) during the study.

**Supplementary Fig. 2.** Serum hormone measurements. A: Measured serum estradiol and progesterone during the menstrual cycle in the control and the CHC group. B: Comparison of serum estradiol and progesterone between the control and the CHC group. C: Comparison of serum estradiol and progesterone between the follicular and the luteal phase of the cycle in the control group. Wilcoxon rank-sum test with continuity correction.