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Role of the Gut Microbiome in Polycystic Ovary Syndrome

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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The Dissertation of Pedro Jahir Torres is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

This thesis is dedicated to all the teachers who told me I could amount to anything, to all the people who were around me and lend a helping hand when I was just trying to study in order to better myself and feed my brain with knowledge, to everyone currently in struggle. You know what I am saying? It's all good baby baby.

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EPIGRAPH

All disease begins in the gut.

-Hippocrates

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ABSTRACT OF THE DISSERTATION

Role of the Gut Microbiome in Polycystic Ovary Syndrome

by

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Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women worldwide. In addition to infertility, many women with PCOS have metabolic abnormalities that result in an increased risk of type 2 diabetes and cardiovascular disease. Although the etiology of PCOS is poorly understood, accumulating evidence suggests that androgen excess is an important contributor to the pathogenesis of PCOS. The human large intestine contains a complex community of microorganisms (the gut microbiome) important for human health and disease. Studies have shown that the gut microbiome is altered in humans with metabolic disorders such as obesity and type 2 diabetes, and that the gut microbiome may be a causal factor in the development of these disorders. Dysbiosis of the gut microbiome has been proposed to factor in the development of PCOS and studies have shown that changes in the gut microbiome are associated with PCOS in women

and in rodent models. However, it is unknown if the gut microbiome plays a causal role in the PCOS phenotype. For this dissertation I used human fecal samples and a letrozole-induced PCOS mouse model together with 16S rRNA gene sequencing to further our understanding of the effects of androgen excess and the role of the gut microbiome in PCOS. Using human fecal samples and patient clinical parameters, the results in chapter 1 suggest that androgen excess may play a critical role in altering the gut microbiome in women with PCOS. Since there is considerable variation in the human gut microbiome due to differences in diet and ethnicity, chapters 2 and 3 used a PCOS mouse model to gain more insight into the pathophysiology of PCOS. The results from chapter 2 indicate that dysregulation of the metabolism and gut microbiome in PCOS may be influenced by the timing of androgen exposure. Lastly, the results from chapter 3 showed that exposure to a healthy gut microbiome improved the metabolic and reproductive phenotype in a PCOS mouse model. Overall, these results suggest that modulating the composition of the gut microbiome may be a potential treatment option for women with PCOS.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS)

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women with an estimated world-wide prevalence of ~10% [1]. PCOS was first described in 1935 by Drs. Irving Stein and Michael Leventhal as a group of patients who demonstrated a combination of hirsutism (male-pattern terminal hair growth in women), chronic anovulation and polycystic ovaries [2, 3]. Since then, there have been several diagnostic criteria proposed for PCOS: 1. The NIH criteria (1990), which requires hyperandrogenism and oligo- or amenorrhea; 2. the Androgen Excess Society criteria (2006), which requires hyperandrogenism and ovulatory dysfunction (e.g., oligo- or amenorrhea and/or polycystic ovaries; and the most widely used PCOS classification 3. the Rotterdam Consensus criteria (2003), which requires at least two of the following: androgen excess (hyperandrogenemia and/or hirsutism), ovarian dysfunction (oligo- or amenorrhea) and/or polycystic ovarian morphology (PCOM) [4]. The most severe clinical manifestation is the classical PCOS phenotype in which women present with both hyperandrogenism and oligo-ovulation irrespective of polycystic ovaries. The next most severe phenotype is ovulatory PCOS in which women present with hyperandrogenism and polycystic ovaries. The non-hyperandrogenic phenotype, which consists of oligo-ovulation and polycystic ovaries, is the least severe phenotype [5].

PCOS Pathophysiology

Although the etiology of PCOS is poorly understood, familial clustering indicates that there is a genetic component [6-8]. Twin studies comparing the correlation of PCOS diagnosis between monozygotic and dizygotic twins have estimated the heritability of PCOS as ~70%, but no clear mode of inheritance has been identified [9]. Altered expression of genes involved

in signal transduction pathways controlling steroidogenesis, steroid hormone action, gonadotropin action and regulation, insulin action and secretion, energy homeostasis and chronic inflammation have been described in PCOS [10-12]. While results have been promising, there are no genes universally accepted as fundamentally important in PCOS pathophysiology. Heritability is commonly assumed to reflect the effects of inherited genomic variation; however, it may also reflect the effects from shared disease-predisposing environments e.g. daughters of a woman with PCOS would be exposed to the same intrauterine environment. Prenatal exposure to androgens in female rodent and primate studies have also implicated the intrauterine environment in PCOS pathogenesis [13-15]. Evidence suggest that the intrauterine environment in pregnant PCOS women is also hyperandrogenic [16]. The clinical manifestations of PCOS often emerge in adolescence or the early reproductive years, suggesting that puberty may be a critical time in the development and pathology of PCOS [17, 18]. Indeed, PCOS has been hypothesized to originate form abnormal pubertal development due to a lack of transition from an androgen-dominated state in early puberty to an estrogenic state in late puberty [19, 20]. Altogether, mounting evidence suggest that PCOS might be a complex multifactorial disorder that arises from interactions among genetic, environmental and intrauterine factors.

Women with PCOS often suffer from profound, long-term health issues [21]. PCOS is the leading cause of anovulatory infertility in women and increases the likelihood of miscarriage and pregnancy complications [22, 23]. In addition, a majority of women with PCOS have abnormalities that increase their risk of developing metabolic disease [4, 24-28]. Obesity is a common finding in women with PCOS and approximately 80% of women with PCOS in the United States are obese [29]. Moreover, obesity exacerbates many of the reproductive and metabolic abnormalities associated with PCOS [30]. Insulin resistance is

another common feature of women with PCOS, which cannot be fully explained by the frequent association with obesity because PCOS women are more insulin resistant than healthy controls matched for body mass index (BMI) [31, 32]. Insulin resistance occurs in 75% of non-obese and 95% of obese women with PCOS [33]. In addition to obesity and insulin resistance, studies have shown that PCOS is associated with abdominal adiposity, hyperinsulinemia, glucose intolerance, and dyslipidemia [34-37]. These associated disorders directly increase the risk of metabolic syndrome, type 2 diabetes, gestational diabetes, non-alcoholic fatty liver disease and cardiovascular disease [25, 38-40]. Studies have shown that hyperandrogenism is strongly correlated with development of a metabolic phenotype [41-43]. Metabolic dysfunction occurs predominately in women diagnosed with hyperandrogenism and ovulatory dysfunction, independent of BMI [7, 44, 45]. Given the limitations of current treatments for metabolic symptoms of PCOS, there is a significant need for studies addressing the development and pathophysiology of the PCOS metabolic phenotype.

Gut Microbiome and PCOS

The human large intestine contains a complex community of microorganism (the gut microbiome) important for human health and disease [46, 47]. Gut microbes offer many benefits to the host including protection against pathogens as well as education of our immune system and integrity of the intestinal barrier [48-50]. The gut microbiome is also involved in the production of short-chain fatty acids via fermentation of dietary soluble fibers, production of essential vitamins such as folic acid and B12 and modification of bile acids, neurotransmitters and hormones [51, 52]. Correlative studies have demonstrated that the gut microbiome of individuals with metabolic disorders, such as obesity and diabetes, differs significantly from healthy individuals [46, 53-57]. Moreover, studies have reported that transplantation of stool from obese donors into germ-free mice results in an obese phenotype,

suggesting that the gut microbiome may play a causative role in metabolic dysregulation [56]. These transplantation studies were complemented with co-housing studies that took advantage of the fact that, since mice are coprophagic, co-housing provides a means for repeated, non-invasive microbial inoculation. Co-housing germ free mice transplanted with stool from obese donors with germ-free mice transplanted with stool from lean donors was shown to protect the mice transplanted with obese donor stool from developing obesity [56, 58, 59]. Altogether, these studies suggest that modulation of the gut microbiome may be a potential treatment option for metabolic disorders.

Dysbiosis of the gut microbiome has been proposed to contribute to the development of PCOS and studies using 16S rRNA gene amplicon sequencing have shown that changes in the gut microbiome are associated with PCOS [46, 60-63]. Women diagnosed with PCOS using the Rotterdam criteria were reported to have a significant reduction in the overall gut bacterial species richness (alpha diversity), shifts in gut microbial composition (beta diversity) and changes in abundance of several bacterial taxa compared to healthy women.

Letrozole Induced PCOS Mouse Model

Since hyperandrogenism is associated with PCOS, researchers have created animal models to study the role of androgens in the development and pathology of PCOS [64-68]. Several mouse models were developed using treatment with exogenous dihydrotestosterone but these models did not exhibit the elevated luteinizing hormone (LH) levels associated with PCOS [69-73]. A PCOS mouse model was developed in pubertal female mice using treatment with the aromatase inhibitor, letrozole, to limit the conversion of testosterone to estrogen which results in increased testosterone and decreased estrogen levels. This model is based on the findings that genetic variants of the aromatase gene are associated with the development of PCOS in women and that a higher androgen/estrogen ratio is found in the ovaries of

women with PCOS [74-78]. Letrozole treatment of pubertal female mice results in the reproductive hallmarks of PCOS including hyperandrogenism, acyclicity, polycystic ovaries, and elevated LH levels [79-81]. This model also exhibits metabolic dysregulation similar to the phenotype in women with PCOS including weight gain, abdominal adiposity, increased fasting blood glucose and impaired insulin levels, impaired glucose stimulated-insulin secretion, insulin resistance and dyslipdemia [79-81]. Letrozole treatment does not alter food intake or energy expenditure, even though locomotion was decreased, suggesting that other mechanisms contribute to the metabolic dysregulation in this model [81]. Similar to women with PCOS, 16S rRNA gene sequencing showed that letrozole treatment was associated with a significant decrease in the alpha diversity and a shift in overall gut microbial composition (beta diversity) [80]. A recent study examining the effects of non-antibiotic drugs on the gut microbiome found that letrozole did not alter the growth of ~40 representatives gut bacteria [82], this suggest that differences in the gut microbial composition found in the PCOS mouse model are not due to a direct effect of letrozole.

The results obtained from the studies presented in this thesis have increased our understanding of the role of hyperandrogenism in shaping the gut microbiome of women with PCOS, how timing of excess androgen exposure may be an important component in the development of the PCOS phenotype and how exposure to a healthy gut microbiome can help improve the PCOS metabolic and reproductive phenotype. All together, these studies provide insight into the pathophysiology of PCOS and suggest that dysbiosis of the gut microbiome may play a causal role in PCOS and that modulation of the gut microbiome may be a potential treatment option for women with this disorder.

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CHAPTER 1

Gut Microbial Diversity in Women with Polycystic Ovary Syndrome Correlates with Hyperandrogenism

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Context: A majority of women with polycystic ovary syndrome (PCOS) have metabolic abnormalities that result in an increased risk of developing type 2 diabetes and heart disease. Correlative studies have shown an association between changes in the gut microbiome and metabolic disorders. Two recent studies reported a decrease in α diversity of the gut microbiome in women with PCOS compared with healthy women.

Objective: We investigated whether changes in the gut microbiome correlated with specific clinical parameters in women with PCOS compared with healthy women. We also investigated whether there were changes in the gut microbiome in women with polycystic ovarian morphology (PCOM) who lacked the other diagnostic criteria of PCOS.

Participants: Subjects were recruited at the Poznan University of Medical Sciences. Fecal microbial diversity profiles of healthy women (n = 48), women with PCOM (n = 42), and women diagnosed with PCOS using the Rotterdam criteria (n = 73) were analyzed using 16S ribosomal RNA gene sequencing.

Results: Lower α diversity was observed in women with PCOS compared with healthy women. Women with PCOM had a change in α diversity that was intermediate between that of the other two groups. Regression analyses showed that hyperandrogenism, total testosterone, and hirsutism were negatively correlated with α diversity. Permutational multivariate analysis of variance in UniFrac distances showed that hyperandrogenism was also correlated with β diversity. A random forest identified bacteria that discriminated between healthy women and women with PCOS.

Conclusion: These results suggest that hyperandrogenism may play a critical role in altering the gut microbiome in women with PCOS. (***J Clin Endocrinol Metab*** **103: 1502–1511, 2018)**

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age, with an estimated worldwide prevalence of 5% to 15% when the Rotterdam consensus criteria are used (1). Hyperandrogenism is a key feature of this disorder, and heritability studies indicate that there is a strong polygenic component (2). PCOS can result in profound, long-term health consequences (3). In addition to increased risks of infertility, miscarriage, and pregnancy complications, many women with PCOS have metabolic abnormalities that increase their risk of developing obesity, type 2 diabetes, and cardiovascular disease (4, 5).

A complex community of microorganisms that is important for human health resides within the large

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Abbreviations: BMI, body mass index; CCA, canonical correspondence analysis; FSH, follicle-stimulating hormone; HOMA-IR, homeostasis model assessment of insulin resistance; LH, luteinizing hormone; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome; PCR, polymerase chain reaction; PD, phylogenetic diversity; PERMANOVA, permutational multivariate analysis of variance; rRNA, ribosomal RNA; SCFA, short-chain fatty acid; SV, sequence variant.

intestine (the gut microbiome) (6). Correlative studies demonstrated that the gut microbiome of individuals with metabolic disorders such as obesity and diabetes differs from that of healthy individuals (7–10). More recently, two studies reported differences in the gut microbiome of Caucasian or Han Chinese women with PCOS, including a decrease in the overall bacterial species richness (α diversity) of the gut microbial community and changes in several bacterial taxa, compared with that of healthy women (11, 12). Fecal microbiome transplantation from obese humans into germ-free mice also resulted in an obese phenotype, indicating a potential causative role of the gut microbiome in the development of metabolic disorders (13, 14).

Given that we previously observed changes in the gut microbiome in a hyperandrogenic mouse model of PCOS (15), we investigated whether changes in the gut microbiome in women with PCOS correlate with hyperandrogenism or other hallmarks of PCOS. We found that women with PCOS had a decrease in biodiversity in the gut microbiome and changes in specific bacterial taxa compared with healthy women. Women with polycystic ovarian morphology (PCOM) also had a change in gut microbial diversity that was intermediate between that of the other two groups. Furthermore, our analyses demonstrated that hyperandrogenism (total testosterone and hirsutism) was correlated with changes in the gut microbiome. A better understanding of the relationship between hyperandrogenism and the gut microbiome in women may lead to new therapeutic approaches for PCOS.

Subjects and Methods

Study cohort

A total of 163 premenopausal women were recruited at the Poznan University of Medical Sciences. Using the Rotterdam criteria, PCOS was diagnosed by the presence of at least two of the following conditions: clinical or biochemical hyperandrogenism (Ferriman-Gallwey score $\geq$8; testosterone level >0.5 ng/mL), oligomenorrhea or amenorrhea (<8 cycles/y), and polycystic ovaries. Congenital adrenal hyperplasia was excluded on the basis of a morning follicular phase 17-hydroxyprogesterone level <2 ng/mL. Diabetes mellitus was excluded on the basis of a fasting glucose level <100 mg/dL and a glucose tolerance test value <200 mg/dL at 30, 60, and 90 minutes and <140 mg/dL at 120 minutes. None of the subjects had elevated prolactin levels, thyroid disease, or Cushing disease. Study participants had no clinical signs or symptoms of any other endocrinopathy, a normal baseline renal function, and normal levels of bilirubin and aminotransferases. Exclusion criteria were the use of oral contraceptives, other steroid hormones, and metformin within the preceding 3 months. Subjects taking antibiotics, probiotics, or laxatives were excluded. All study participants were at least 18 years old and provided informed consent. The study was approved by the institutional review boards at the Poznan University of Medical Sciences and the University of California, San Diego.

Sampling and laboratory measurements

The study visits took place between 8:30 and 11 AM. Clinical assessments included determination of body mass index (BMI) and hirsutism. Venous blood was collected after an overnight fast, and serum was stored at −80°C until the analyses were performed. A 2-hour oral glucose tolerance test was performed with determinations of glucose and insulin in the fasting state as well as after a 75-g glucose load at 30, 60, 90, and 120 minutes. Fecal samples were collected from the rectum using a cotton swab (CultureSwab 220135; Becton Dickinson). Samples were stored at −80°C within 20 minutes of collection. Transvaginal ultrasonographic evaluations were performed using the Aloka ProSound 7 (Aloka Co. Ltd.). The ovaries were measured in three perpendicular diameters. Ovarian volume was determined using the prolate ellipsoid formula. Glucose levels were determined using the enzymatic reference method with hexokinase. Serum testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), sex hormone-binding globulin, and insulin levels were determined using electrochemiluminescence assays (Roche Cobas 6000 System).

DNA isolation

Rectal swab samples were shipped on dry ice to the University of California, San Diego and stored at −80°C. Genomic DNA was extracted from samples in a class II biological safety cabinet using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc.). Solution C1 (60 μL) was added to the bead beating tubes, and the cotton tips of the swabs were broken off directly into the tubes. Tubes were vortexed at maximum speed for 15 minutes using the PowerSoil Vortex Adaptor (MoBio Laboratories, Inc). The remaining steps were performed as directed by the manufacturer. Genomic DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific), and the DNA was stored at −80°C.

16S ribosomal RNA amplicon sequencing

For each sample, the V4 hypervariable region of the 16S ribosomal RNA (rRNA) gene was polymerase chain reaction (PCR) amplified with primers 515F and 806R (16). The reverse primers contained unique 12-base-pair Golay barcodes that were incorporated into the PCR amplicons (17). PCR parameters were as follows: denaturing at 94°C for 3 minutes followed by amplification for 35 cycles at 94°C for 45 seconds, at 50°C for 60 seconds, at 72°C for 90 seconds, and with a final extension of 72°C for 10 minutes. Amplicon sequence libraries were prepared at The Scripps Research Institute Next Generation Sequencing Core Facility, where the libraries were sequenced on an Illumina MiSeq as previously described (15).

16S rRNA amplicon analysis

Raw sequences were imported into QIIME 2 (v.2017.6; https://docs.qiime2.org/2017.6/) (18) using the q2-tools-import script, and sequences were demultiplexed using the q2-demux emp-single script. This resulted in 5.6 million sequences with an average of 30,000 sequences per sample. The 16S rRNA sequences generated in this study were deposited into the European Nucleotide Archive (study accession no. PRJEB22972). DADA2 software was used to obtain a set of observed sequence variants (SVs) (19). DADA2 uses an Illumina sequence error correction algorithm to derive an abundance distribution of distinct SVs, which can differ by only a single nucleotide. On the

basis of the quality scores, the forward reads were truncated at position 220 using the q2-dada2-denoise script. Taxonomy was assigned using a pretrained naive Bayes classifier [Greengenes 13_8 99% operational taxonomic units (OTUs)], and the q2-feature-classifier plug-in (20). Singletons and SVs present in <10% of samples were removed to minimize the effect of spurious, low abundance sequences using the q2-feature-table filter-features script. The resulting SVs were then aligned using MAFFT (21), and a phylogenetic tree was built using FastTree (22). Taxonomic distributions of the samples were calculated using the q2-taxa-barplot script. The rectal swabs contained bacteria representative of fecal samples, and there was no evidence of contamination with oral, skin, or vaginal bacteria.

α and β diversity metrics were computed using the q2-diversity core-metrics script at a rarefied sampling depth of 500. Rarefaction resulted in the removal of three samples (one control and two PCOS) that had <500 sequences per sample. Four α diversity metrics, observed SVs, Faith phylogenetic diversity (PD), Shannon, and Pielou were used to estimate fecal microbial community richness, PD, information content, and evenness, respectively (23–25). Two outliers (both PCOS) were identified in the measures of α diversity and removed from the analyses. UniFrac was used to compare the similarity (β diversity) among the microbial communities by calculating the shared PD between pairs of microbial communities (26). A cluster of outliers (four control and four PCOS) was identified in the principal coordinates analysis (PCoA) of unweighted UniFrac and removed from the analyses.

Statistical analysis

Statistical calculations were performed in the RStudio statistical package (version 0.99.893). Data were tested for normality via the Shapiro-Wilk test. Variables that were not normally distributed were transformed or ranked. Differences in the clinical characteristics of study participants and microbiome characteristics were analyzed using one-way or multifactor analysis of variance followed by a Tukey honest significant difference test for multiple *post hoc* comparisons. Multivariable linear regression models were generated by backward stepwise elimination implemented in R using the "step" library. Simple linear regression and Pearson rank correlation were also performed. PCoA and canonical correspondence analysis (CCA) plots were constructed using the phyloseq R package (V.1.19.1). PCoA plots were used to represent the similarity of fecal microbiome samples on the basis of multiple variables in the data set, whereas CCA was used to visualize the relationship of the fecal microbiome with specific clinical parameters. Permutational multivariate analysis of variance (PERMANOVA) used unweighted Unifrac distance measures to assess bacterial community compositional differences and relationships to patient clinical characteristics (999 permutations; "vegan" package). CCA combined with PERMANOVA was performed to single out significant variables driving microbiome composition and to orient the data for visualizing the differences among the factors of interest. A random forest classifier (27) was implemented in R using the "randomForest" library to identify α diversity factors and bacterial observed SVs that discriminate between healthy women and women with PCOS. Since random forest assumes that there are equal samples in each group, we sampled a random subset of the samples from the women with PCOS to compare with the control group.

Results

Clinical characteristics of study participants

Gut microbial diversity profiles were generated for a total of 163 women: 48 healthy controls, 42 with PCOM,

Table 1. Clinical Characteristics of Study Participants

	Diagnosis			ANOVA	Tukey HSD (Adjusted for Multiple Comparisons)		
	Control (n = 48)	PCOM Only (n = 42)	PCOS (n = 73)	*P* Value	Control vs PCOM	Control vs PCOS	PCOM vs PCOS
Age, y	29.4 ± 4.9	29.8 ± 5.3	27.4 ± 4.9	0.04	0.97	0.11	0.08
BMI, kg/m^2	23.7 ± 4.1	22.6 ± 4.2	25.6 ± 6.5	0.02	0.34	0.39	0.01
Testosterone, ng/mL	0.3 ± 0.1	0.3 ± 0.1	0.56 ± 0.2	<0.0001	0.98	<0.0001	<0.0001
Free testosterone, V	0.35 ± 0.2	0.3 ± 0.2	0.9 ± 0.5	<0.0001	0.85	<0.0001	<0.0001
Hirsutism (Ferriman-Gallwey)	2.9 ± 1.3	3.5 ± 1.8	8.1 ± 4.3	<0.0001	0.31	<0.0001	<0.0001
Menses per y	12.1 ± 0.5	10.9 ± 1.8	8.1 ± 3.4	<0.0001	0.07	<0.0001	<0.0001
LH, IU/L	7.7 ± 5.8	10.8 ± 14.3	11.9 ± 8.4	<0.0001	0.13	<0.0001	0.02
FSH, IU/L	5.7 ± 1.9	6.4 ± 2.9	5.5 ± 1.9	0.29	0.52	0.91	0.25
LH/FSH ratio	1.4 ± 0.6	1.5 ± 0.8	2.3 ± 1.4	<0.0001	0.59	<0.0001	0.0006
Fasting glucose, mmol/L	4.86 ± 0.34	4.85 ± 0.4	5.14 ± 1.87	0.37	1	0.47	0.47
Fasting insulin, pmol/L	48.5 ± 18.5	46.6 ± 21.9	61.4 ± 38.2	0.05	0.83	0.2	0.06
HOMA-IR	1.75 ± 0.7	1.69 ± 0.84	2.27 ± 1.54	0.01	0.97	0.05	0.03
Hyperandrogenism	0	0	62	<0.0001	1	<0.0001	<0.0001
Oligomenorrhea	0	0	34	<0.0001	1	<0.0001	<0.0001
PCOM	0	42	71	<0.0001	<0.0001	<0.0001	0.39

Data are presented as mean ± standard deviation. Nonnormal data were ranked and analyzed by one-way analysis of variance followed by the Tukey HSD test.

Abbreviations: HSD, honest significant difference; V, Vermeulen equation.

and 73 diagnosed with PCOS. Of the 73 women with PCOS, 21 had all three criteria (hyperandrogenism, oligomenorrhea, and PCOM), 39 had hyperandrogenism and PCOM, two had hyperandrogenism and oligomenorrhea, and 11 had oligomenorrhea and PCOM. Table 1 summarizes the clinical characteristics of the study participants. Compared with healthy women in the control group and those with PCOM, the cohort of women with PCOS had higher levels of serum total testosterone and free testosterone as well as an increase in hirsutism and a decrease in the number of menses per year. Women with PCOS also had increased levels of serum LH, an increased ratio of LH/FSH, but no detectable change in serum FSH levels. In addition, although fasting glucose and insulin levels were not different, women with PCOS had higher homeostasis model assessment of insulin resistance (HOMA-IR) values. Although age and BMI did not differ between controls and women with PCOS, there was a small difference in BMI between women with PCOM and women with PCOS.

PCOS was associated with reduced biodiversity in the gut microbiome

In total, 481 observed SVs (analogous to OTUs) were identified from the rectal swab samples. Women with PCOS had reduced gut microbiome α diversity compared with healthy women as measured by abundance (observed SVs; $P = 0.04$) and PD (Faith PD; $P = 0.02$) [Fig. 1(a) and 1(b)]. Women with PCOM displayed an intermediate phenotype in both observed SVs and Faith PD because the α diversity of their gut microbiome was not statistically different from that of healthy women or women with PCOS. Women with PCOS also tended to have lower Shannon diversity, which accounted for both abundance and evenness of SVs, than controls ($P = 0.1$) [Fig. 1(c)]. There was no difference in the evenness of the gut microbiome of women with PCOS compared with that of either controls or women with PCOM [Fig. 1(d)].

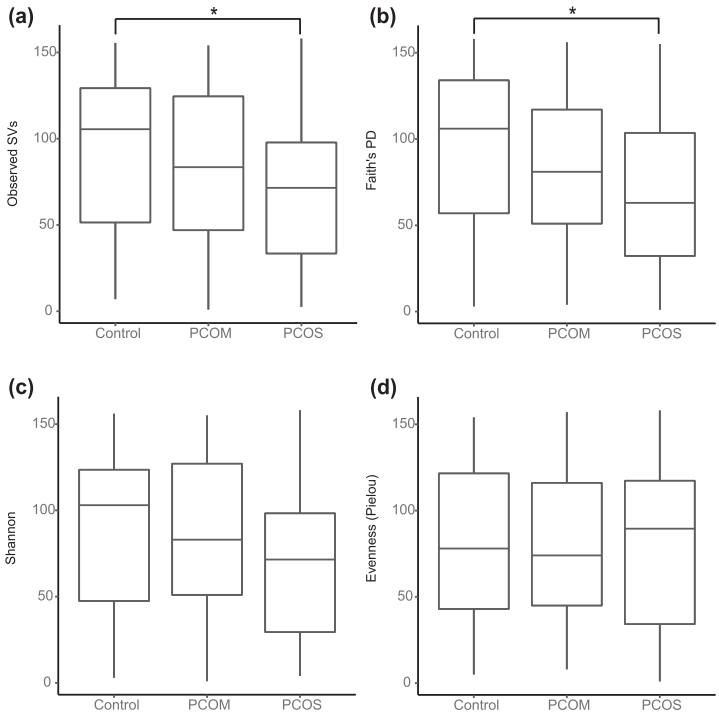

Figure 1. Biodiversity of the gut microbiome was decreased in women with PCOS. Box plots of α diversity in fecal samples from healthy women (controls; n = 47), women with PCOM (n = 41), and women diagnosed with PCOS using the Rotterdam criteria (n = 70) are shown, with whiskers extending 1.5× past the interquartile range. (a–d) α diversity was calculated using (a) the number of observed SVs as an estimate of species richness, (b) Faith PD as an estimate of species richness that takes phylogenetic relationships into account, (c) Shannon as an estimate of both species richness and evenness, and (d) Pielou as an estimate of the evenness of a community. One-way analysis of variance was performed on ranked data with the Tukey honest significant difference *post hoc* test to compare means among groups. $*P < 0.05$.

Table 2. Summary of Multiple Regression Analysis Relating Patient Parameters to α Diversity

	Std. Error	*t* Value	*P* Value
Observed SVs			
Control vs PCOM	9.61	−1.49	0.13
Control vs PCOS	18.35	−2.28	0.02[a]
Testosterone, ng/mL	24.50	−2.58	0.01[a]
Hirsutism	1.25	−1.70	0.09
Hyperandrogenism	18.67	−2.28	0.02[a]
Menses per y	2.27	−2.05	0.04[a]
Oligomenorrhea	16.67	−1.51	0.13
LH/FSH ratio	3.20	1.75	0.08
Faith PD			
Control vs PCOM	37.14	−1.79	0.07
Control vs PCOS	48.13	−2.55	0.01[a]
Testosterone, ng/mL	24.38	−2.00	0.04[a]
Hirsutism	1.25	−2.17	0.03[a]
Hyperandrogenism	18.43	−3.25	0.001[b]
Menses per y	1.56	−1.57	0.11
PCOM	43.77	1.42	0.16
LH/FSH ratio	3.17	1.76	0.08

Data were ranked and a backward stepwise regression was used to select the model that best predicted observed SVs ($r = 0.14$) and Faith PD ($r = 0.16$).

[a] $P < 0.05$.

[b] $P < 0.01$.

Higher total testosterone levels and hirsutism correlated with lower biodiversity in the gut microbiome

Backward stepwise regression was used to build multiple regression models for identification of clinical parameters that best predicted two measures of α diversity (*i.e.*, observed SVs and Faith PD). Table 2 shows the factors that contributed to the multiple regression models. Total testosterone level, hyperandrogenism, and number of menses per year correlated with observed SVs in the multiple regression model, whereas total testosterone level, hirsutism, and hyperandrogenism correlated with Faith PD. The ratio of LH/FSH may also correlate with α diversity (observed SVs and Faith PD; $P = 0.08$). In contrast, age, BMI, and HOMA-IR did not correlate with α diversity and were not included in the models. In addition, simple linear regression was performed on clinical or biochemical hyperandrogenism to get a better understanding and visualization of the data. Both serum total testosterone level and hirsutism showed negative correlations with observed SVs ($P = 0.006$ and $P = 0.02$, respectively) and Faith PD ($P = 0.05$ and $P = 0.03$, respectively) (Fig. 2).

Hyperandrogenism was associated with changes in the gut microbiome

β *diversity*

In addition to assessing α diversity, we used unweighted and weighted UniFrac analyses to compare the similarity of the gut microbial communities (β diversity) between healthy women and women with PCOS. α diversity estimates the within-sample biodiversity, whereas β diversity estimates the biodiversity between samples. PCoA and PERMANOVA were used to analyze the relationship between overall gut bacterial composition and clinical characteristics of the study participants. Although there was no distinct clustering between samples from controls and those from women with PCOS [Fig. 3(a)], PERMANOVA tests detected a highly significant effect of hyperandrogenism ($P = 0.0009$) and, to a lesser extent, diagnostic group ($P = 0.08$) on the microbial community composition (Table 3). When CCA was applied to visualize the relationship of the gut microbial community structure to clinical and biochemical hyperandrogenism, a separation between samples from controls and from women with PCOS that correlated with hirsutism was observed ($P = 0.06$) [Fig. 3(b)].

Random forest identified bacterial taxa that distinguished between healthy women and women with PCOS

The random forest machine learning classifier was trained to determine how well healthy women and women with PCOS could be predicted on the basis of α diversity and bacteria represented by observed SVs. The random forest had the highest accuracy in distinguishing women with PCOS (65% accuracy) followed by controls (50% accuracy). The variable importance by mean decrease in accuracy was then calculated from the random forest model (Supplemental Fig. 1). The relative abundance of the eight bacterial genera whose removal caused the greatest decrease in model accuracy (*i.e.*, the most important for classification) was graphed for healthy women and women with PCOS [Fig. 3(c)]. These bacteria included *Porphyromonas* spp., *Bacteroides coprophilus*, *Blautia* spp., *Faecalibacterium prausnitzii*, *Anaerococcus* spp., *Odoribacter* spp., *Roseburia* spp., and *Ruminococcus bromii*.

Discussion

This study demonstrated that Caucasian women diagnosed with PCOS using the Rotterdam criteria had a reduction in overall species richness (α diversity) of the gut microbiome compared with that of healthy women and changes in the composition of the microbial community (β diversity). Interestingly, our study found that the biodiversity of the microbiome strongly correlated with hyperandrogenism. More specifically, observed SVs and Faith PD were both negatively correlated with total testosterone level and hirsutism, whereas hyperandrogenism had a highly significant effect on the structure of the bacterial community as measured by

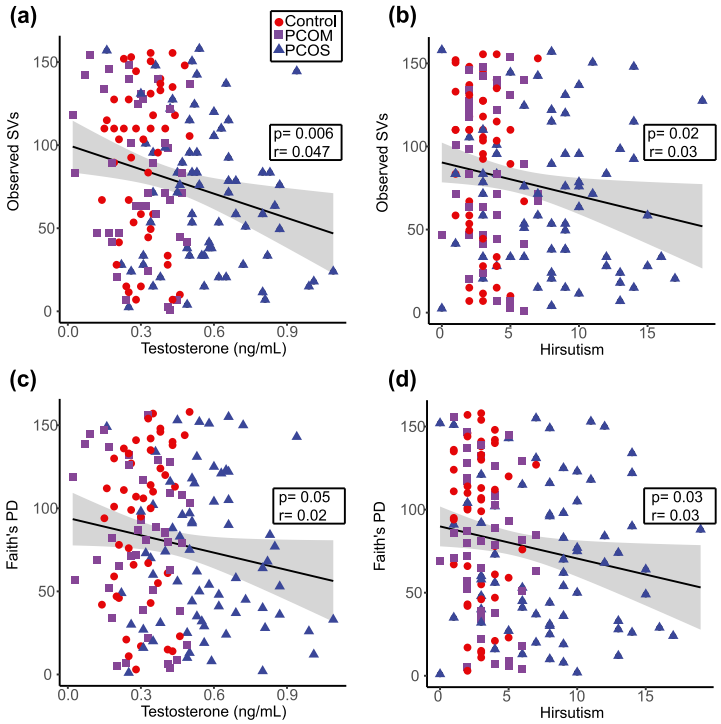

Figure 2. A decrease in gut bacterial biodiversity correlated with an increase in testosterone level and hirsutism. (a–d) Scatterplots and trend lines show the relationship between testosterone and (a) observed SVs or (c) Faith PD as well as the relationship between hirsutism and (b) observed SVs or (d) Faith PD. Results of a Pearson correlation (*P* value and correlation coefficient) are shown in the insets, with the gray shaded areas indicating the 95% confidence interval for the line of best fit. Healthy women (controls; n = 47), women with PCOM (n = 41), and women with PCOS (n = 70).

unweighted UniFrac. We also observed an intermediate phenotype for women with PCOM regarding gut microbiome α diversity, suggesting that further studies are warranted to determine whether the gut microbiome of women with PCOM is significantly altered compared with that of healthy women.

α diversity metrics estimate the overall biodiversity of a community (*i.e.*, the bacterial species in the gut microbiome). Compared with healthy controls, women with PCOS had a reduced overall number of bacterial species and lower PD (observed SVs and Faith PD), whereas there was no difference in community evenness (Pielou) [Fig. 1(a)–1(d)]. This agrees with two previous studies that found a decrease in α diversity in women with PCOS compared with healthy women (11, 12). Reduced α diversity of the gut microbiome was also observed in humans with metabolic diseases compared with healthy individuals. Indeed, lower α diversity of the gut microbiome was consistently associated with human obesity according to several recent meta-analyses (28–30).

In the field of ecology, species richness has been proposed to correlate with the health of an ecosystem, as diverse communities may increase the stability and productivity of an ecosystem (31). In terms of the gut microbiome, it is possible that decreased bacterial diversity results in changes in gut function that can exacerbate diseases, including PCOS, though much work remains to be done to understand how changes in the gut microbiome influence host physiology.

Multiple and single linear regression analyses showed that the decrease in α diversity was associated with total testosterone level and hirsutism [Fig. 2(a)–2(d); Table 2]. These results concur with the negative correlation we observed between α diversity and testosterone level in a hyperandrogenic, letrozole-induced PCOS mouse model (15). Interestingly, factors such as the number of menses per year and the LH/FSH ratio also contributed to the multiple regression models but did not have a significant association with α diversity in the single linear regression analysis. In contrast, free testosterone, LH, and FSH

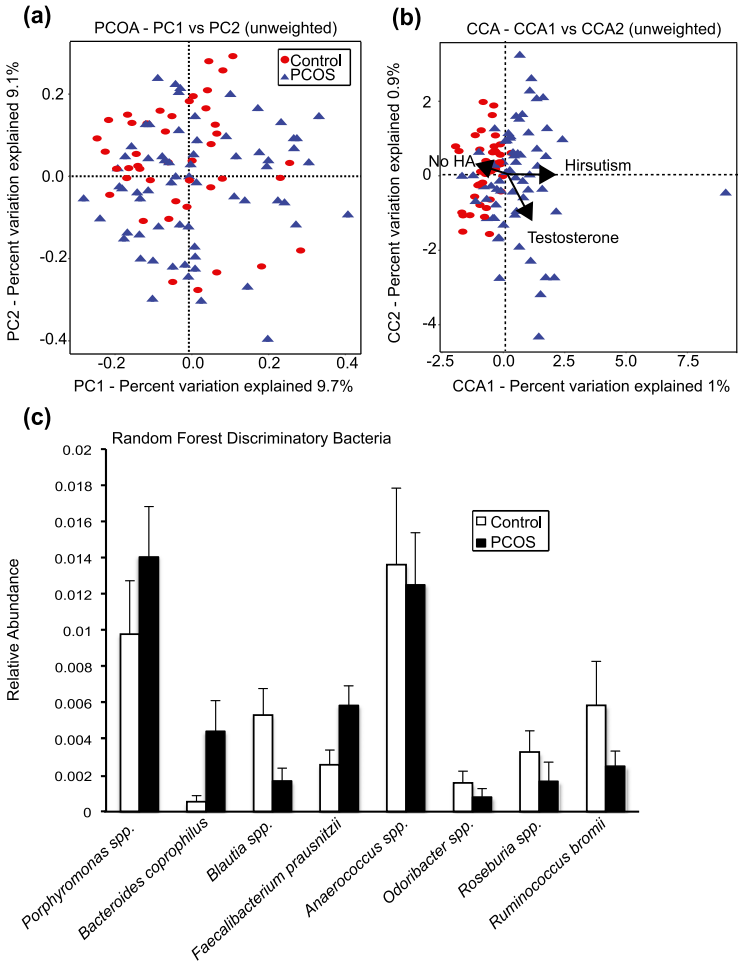

Figure 3. β diversity of the gut bacterial community was influenced by hyperandrogenism (HA), and random forests identified bacterial taxa that distinguished between healthy women and women with PCOS. (a) PCoA of β diversity (unweighted UniFrac distances) of fecal samples from healthy women (controls; n = 43) and women with PCOS (n = 66). Proportion of variance explained by each principal coordinate (PC) axis is denoted on the corresponding axis. Permutation analysis of variance of the unweighted UniFrac distances indicated that hyperandrogenism had a strong influence on the gut microbial community (P = 0.0009). (b) CCA represents the relationship between β diversity and specific variables of interest, such as HA, testosterone, and hirsutism. CCA demonstrated that changes in the gut microbial communities between healthy women and women with PCOS correlated with hirsutism (permutation test; P = 0.06). Proportion of variance explained by each CCA axis is denoted on the corresponding axis. The arrows represent the direction and strength of the correlation between gut microbiome composition and specific variables of interest. (c) A random forest classifier was used to identify bacterial observed SVs that best distinguished between healthy women (controls) and women with PCOS. The relative mean abundances (mean ± standard error of the mean) of the top eight most discriminant observed SVs are identified to the genus and species level when possible.

levels, as well as age, BMI, or HOMA-IR, did not contribute to the multiple regression models or correlate with α diversity.

In addition to α diversity, our study demonstrated a difference in the overall gut microbial composition (β diversity) between healthy women and women with PCOS. Our results agree with the two aforementioned studies that demonstrated changes in β diversity between healthy women and women with PCOS according to unweighted Unifrac and Bray-Curtis analyses (11, 12). In addition, using PERMANOVA, we demonstrated that hyperandrogenism was strongly correlated with changes in the gut microbiome (Table 3). CCA also identified a difference between the gut microbiome of women with

Table 3. Summary of Permutational Analysis of Variance Assessing the Effect of Patient Parameters on Unweighted UniFrac Distances (β Diversity)

	Mean Squares	Pseudo-*F*	*P* Value
Control vs PCOS	0.31	1.33	0.08
Age, y	0.22	1.01	0.42
BMI, kg/m^2	0.18	0.81	0.79
Testosterone, ng/mL	0.24	1.09	0.29
Hirsutism	0.18	0.81	0.77
Menses per y	0.24	1.08	0.31
LH/FSH ratio	0.19	0.85	0.71
Fasting glucose, mmol/L	0.24	1.1	0.37
Fasting insulin, pmol/L	0.21	0.97	0.51
HOMA-IR	0.19	0.89	0.67
Hyperandrogenism	0.47	2.11	0.0009[a]
Oligomenorrhea	0.21	0.96	0.52
PCOM	0.20	0.95	0.52

[a] $P < 0.001$.

PCOS and that of healthy women and showed that hirsutism was associated with the observed compositional differences ($P = 0.06$) [Fig. 3(b)]. Our results agree with the study by Liu *et al.* (12), who used SparCC to analyze the gut microbiome of Han Chinese women with PCOS and healthy women and found that a number of distinct bacterial OTUs correlated with both total testosterone level and hirsutism. Although one cannot infer causation from association studies, the accumulating data from studies of humans and rodent models suggest that androgen levels may have a significant effect on the composition of the gut microbiome in women with PCOS.

Kruskal-Wallis tests did not detect significant differences between the relative abundance of specific bacterial taxa in the gut microbiome of healthy women compared with women with PCOS after correction for multiple comparisons. However, a supervised learning approach using the random forest method identified several bacteria that distinguished the gut microbiome of healthy women from that of women with PCOS [Fig. 3(c)]. The relative abundance of *Porphyromonas* spp., *B. coprophilus, Blautia* spp., and *F. prausnitzii* was consistently higher in women with PCOS, whereas *Anaerococcus* spp., *Odoribacter* spp., *Roseburia* spp., *and R. bromii* were lower [Fig. 3(c)]. *Porphyromonas* has been reported to increase gut permeability and dysbiosis (32). The relative abundance of *B. coprophilus* was reported to be higher in obese individuals (33), whereas patients with type 2 diabetes and glucose intolerance had greater numbers of *Blautia* (34). Interestingly, *F. prausnitzii* is a commensal bacterium known to produce short-chain fatty acids (SCFAs), and in several reports, lower abundance of this bacterium was associated with obesity and Crohn disease (35), which is opposite to the pattern we observed in women with PCOS [Fig. 3(c)].

The four taxa identified by random forest that had lower abundance in women with PCOS [Fig. 3(c)] are all known to synthesize SCFAs. SCFAs are microbial metabolites that have distinct physiological effects on the host. Butyrate, in particular, is involved in a number of beneficial processes to the host, including downregulation of bacterial virulence; maintenance of colonic homeostasis, including acting as an energy source for intestinal epithelial cells; and anti-inflammatory effects (36). Decreased levels of certain strains of *Odoribacter* and *Roseburia* have been associated with Crohn disease and ulcerative colitis and were thought to increase the host's inflammatory response via reduced SCFA production (37, 38). Specific strains of *Anaerococcus* are more abundant in obese individuals (33), whereas *R. bromii* was associated with a lower concentration of SCFAs and insulin sensitivity (39, 40).

Conclusion

In summary, our study demonstrated that hyperandrogenism was correlated with changes in the gut microbiome in women with PCOS. Our findings suggest that androgens may be an important factor in shaping the gut microbiome and that changes in the gut microbiome may influence the development and pathology of PCOS. If hyperandrogenism drives the microbial composition of the gut, it would be interesting to determine if treatment of PCOS with androgen antagonists or oral contraceptives results in recovery of the gut microbiome and improvement of the PCOS metabolic phenotype. Moreover, it would be informative to determine whether the gut microbiome of women diagnosed with PCOS using the criteria of oligomenorrhea and polycystic ovaries is distinct from that of women diagnosed with the other subtypes of PCOS that include hyperandrogenism.

Although many studies have reported that obesity was associated with changes in the gut microbiome, it is noteworthy that BMI or HOMA-IR did not correlate with changes in α or β diversity of the gut microbiome in our study. One possible explanation is that the average BMI of the women in this study was 24.32 ± 0.85 kg/m^2. Further sampling of the gut microbiome of obese women with or without PCOS could address whether obesity and insulin resistance influence the gut microbiome in women with PCOS. However, given variations in the human gut microbiome, large clinical cohorts will likely be needed to address these questions. Future studies to determine whether specific gut bacterial species play a causative role in PCOS will also be important in determining whether probiotics are a treatment option for PCOS.

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CHAPTER 2

Letrozole Treatment of Adult Female Mice Results in a Similar Reproductive Phenotype but Distinct Changes in Metabolism and the Gut Microbiome Compared to Pubertal Mice

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Letrozole treatment of adult female mice results in a similar reproductive phenotype but distinct changes in metabolism and the gut microbiome compared to pubertal mice

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Abstract

Background: A majority of women with polycystic ovary syndrome (PCOS) have metabolic dysfunction that results in an increased risk of type 2 diabetes. We previously developed a pubertal mouse model using the aromatase inhibitor, letrozole, which recapitulates many of the reproductive and metabolic features of PCOS. To further our understanding of the effects of androgen excess, we compared the effects of letrozole treatment initiated in puberty versus adulthood on reproductive and metabolic phenotypes as well as on the gut microbiome.

Results: Letrozole treatment of both pubertal and adult female mice resulted in reproductive hallmarks of PCOS, including hyperandrogenemia, anovulation and polycystic ovaries. However, unlike pubertal mice, treatment of adult female mice resulted in modest weight gain and abdominal adiposity, minimal elevation in fasting blood glucose and insulin levels, and no detectable insulin resistance. In addition, letrozole treatment of adult mice was associated with a distinct shift in gut microbial diversity compared to letrozole treatment of pubertal mice.

Conclusions: Our results indicate that dysregulation of metabolism and the gut microbiome in PCOS may be influenced by the timing of androgen exposure. In addition, the minimal weight gain and lack of insulin resistance in adult female mice after letrozole treatment indicates that this model may be useful for investigating the effects of hyperandrogenemia on the hypothalamic-pituitary-gonadal axis and the periphery without the influence of substantial metabolic dysregulation.

Keywords: Gut microbiome, Polycystic ovary syndrome, Hyperandrogenism, Puberty

Background

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-aged women with an estimated world-wide prevalence of 6–15%, but the etiology of PCOS is not well understood [1]. Heritability and twin studies have identified a strong genetic component that is likely polygenic [2–4]. Recent genome-wide association studies have reported multiple susceptibility loci associated with an increased risk of developing PCOS [5]. Environmental factors, such as prenatal exposure to androgens may also play a role in the etiology of PCOS [6]. Currently, diagnosis is made using the Rotterdam Consensus criteria (2003), which require at least two of the following: hyperandrogenism, oligo- or amenorrhea and polycystic ovaries [1].

Studies have shown that women with PCOS often suffer from profound, long-term health issues [7]. PCOS is the leading cause of anovulatory infertility in women and increases the likelihood of miscarriage and pregnancy complications [8, 9]. In addition, a majority of

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women with PCOS have abnormalities that increase their risk of developing metabolic disease [1, 10–15]. A large, retrospective study demonstrated that PCOS was associated with an increased risk of obesity (16 vs. 3.7%) and type 2 diabetes (12.5 vs. 3.8%) over a 15-year period [16]. Studies show that hyperandrogenism is strongly correlated with development of a metabolic phenotype. Metabolic dysfunction occurs predominantly in women diagnosed with hyperandrogenism and ovulatory dysfunction, independent of body mass index [17, 18].

A complex community of microorganisms (the microbiome) resides within the large intestine and is important for human health [19, 20]. Correlative studies have demonstrated that the gut microbiome of individuals with metabolic disorders, such as obesity and diabetes, differ significantly from healthy individuals [21–25]. In addition, mouse models of obesity are associated with gut microbiome dysregulation [26–31]. Studies have also shown that fecal transplantation of the gut microbiome from obese individuals into germ-free mice results in an obese phenotype [22, 32, 33], indicating a potential role of the gut microbiome in the development of metabolic disorders [34]. Recent studies indicate that changes in the gut microbiome are associated with PCOS. Women diagnosed with PCOS using the Rotterdam criteria were reported to have a significant reduction in the overall bacterial species richness (alpha diversity) of the gut microbial community and changes in the abundance of several bacterial taxa compared to healthy women [35–37]. Interestingly, a study from our lab also showed a significant correlation between hyperandrogenism and diversity of the gut microbiome, suggesting that androgens may influence the composition of the gut microbiome in women [37].

Since hyperandrogenism is associated with PCOS, researchers have created animal models to study the role of androgens in the development and pathology of PCOS [reviewed in [38–42]]. Several mouse models were developed using treatment with exogenous dihydrotestosterone but these models did not exhibit the elevated LH levels associated with PCOS [43–47]. We developed a PCOS mouse model in pubertal female mice using treatment with the aromatase inhibitor, letrozole, to limit the conversion of testosterone to estrogen which results in increased testosterone and decreased estrogen levels. This model is based on the findings that genetic variants of the aromatase gene are associated with the development of PCOS in women and that a higher androgen/estrogen ratio is found in the ovaries of women with PCOS [48–52]. We demonstrated that this mouse model has many hallmarks of PCOS including hyperandrogenemia, elevated LH levels, acyclicity, and polycystic ovaries [53, 54]. This model also exhibited a metabolic phenotype including weight gain, abdominal adiposity, dysglycemia, hyperinsulinemia, and insulin resistance after 5 weeks of letrozole treatment [55]. Similar to women with PCOS, we also showed that there was a significant decrease in the alpha diversity of the gut microbiome in the letrozole-induced PCOS mouse model that correlated with hyperandrogenism [54]. To gain more insight into the effects of androgen excess, we investigated whether the timing of testosterone exposure was important for the pathophysiology of PCOS by evaluating the effects of letrozole treatment on reproductive and metabolic phenotypes in pubertal versus adult female mice.

Results

Letrozole treatment of adult female mice resulted in reproductive hallmarks of PCOS

In this study, we investigated whether the age at which letrozole treatment was initiated affected development of the PCOS phenotype in female mice (Fig. 1a). Five weeks of letrozole treatment in pubertal and adult female mice resulted in elevated serum testosterone levels (Fig. 1b-c). Letrozole treatment in adult female mice also resulted in increased LH levels (Fig. 1d) and acyclicity (Fig. 1e). Interestingly, the ovarian weight was similar in placebo and letrozole-treated adult mice (Fig. 1f). This is in contrast to the increase in ovarian weight previously observed in letrozole-treated pubertal mice [53, 54]. Similar to pubertal mice, letrozole treatment of adult female mice resulted in ovaries with cystic follicles and hemorrhagic cysts (Fig. 1g). Ovaries in the letrozole-treated mice also lacked corpora lutea, indicating a lack of ovulation compared to placebo-treated mice.

Letrozole treatment of adult female mice resulted in minimal weight gain and abdominal adiposity after 5 weeks of treatment

Similar to previous reports [53, 54], letrozole treatment of pubertal female mice for 2 weeks resulted in substantial weight gain compared with placebo treatment, and weight was still increased at the end of the study (Fig. 2a). In contrast, letrozole treatment of adult female mice resulted in a more modest weight gain after 2 weeks of treatment and weight was not statistically different compared to placebo-treated mice after 5 weeks of treatment (Fig. 2a). Letrozole treatment of pubertal female mice resulted in a significant change in abdominal adiposity compared with placebo as reflected in an increase in the weight of the parametrial fat pad relative to total body weight (Fig. 2b). However, letrozole treatment of adult mice did not result in increased abdominal adiposity compared with placebo-treated mice (Fig. 2b).

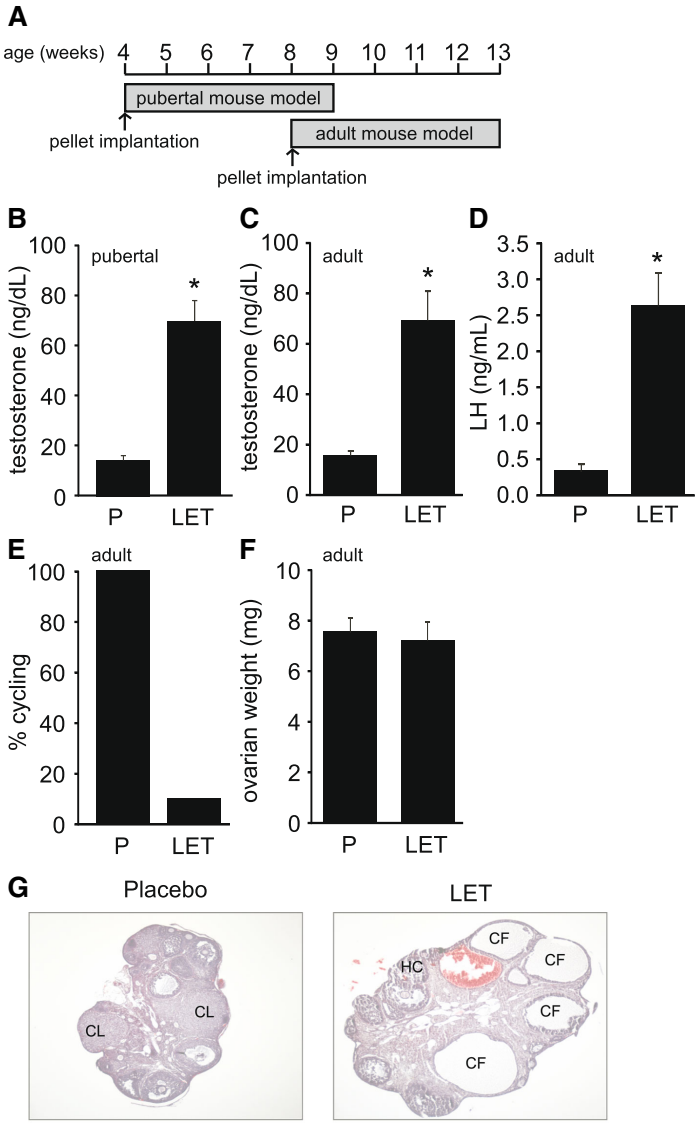

Fig. 1 Letrozole treatment of adult female mice resulted in reproductive hallmarks of PCOS. Letrozole (LET) treatment was initiated at 8 weeks of age in the adult PCOS mouse model compared to 4 weeks of age in the pubertal PCOS mouse model (**a**). LET treatment of pubertal and adult female mice for 5 weeks resulted in elevated serum testosterone (**b-c**). LET treatment of adult female mice resulted in elevated LH levels (**d**), and decreased cyclicity as measured by percentage (%) of mice that had an estrous cycle between 4 and 5 weeks of treatment (**e**). In contrast to pubertal mice, LET treatment of adult female mice did not result in an increase in ovarian weight (**f**). Pubertal PCOS model ($n = 24$ placebo (P), $n = 22$ LET; adult PCOS model ($n = 16$ P, $n = 14$ LET). Student t-test; * $p < 0.05$. LET treatment of adult female mice resulted in ovaries lacking corpora lutea (CL) and containing cystic follicles (CF) and hemorrhagic cysts (HC) compared to placebo-treated mice (**g**)

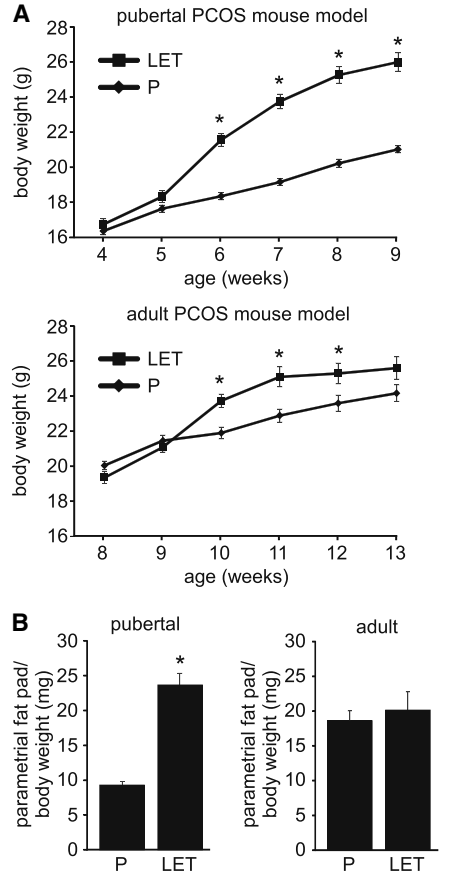

Fig. 2 Five weeks of letrozole treatment of adult female mice did not result in substantial weight gain or abdominal adiposity. The phenotype of pubertal (4 week-old) versus adult (8 week-old) female mice treated with placebo (P) or letrozole (LET) for 5 weeks was compared. In contrast to pubertal mice, 5 weeks of LET treatment of adult female mice did not result in a significant increase in weight (**a**) or abdominal adiposity as measured by parametrial fat relative to total body weight (**b**). Pubertal PCOS model (n = 24 P, n = 22 LET; adult PCOS model (n = 16 P, n = 14 LET). Student *t*-test, * $p < 0.05$

Letrozole treatment of adult female mice resulted in less elevation of fasting blood glucose and insulin levels and did not result in insulin resistance

Both the pubertal and adult PCOS mouse models displayed dysglycemia and hyperinsulinemia but the phenotype was more modest in the adult model. Letrozole treatment of pubertal female mice resulted in elevated fasting blood glucose (FBG) levels and a 3-fold increase in fasting blood insulin levels (Fig. 3a-b). In contrast, letrozole treatment of adult female mice resulted in a slight but statistically significant increase in FBG and a 2-fold increase in insulin levels. There was no significant difference in the response to exogenous glucose in a glucose tolerance test in mice treated with letrozole compared to placebo in either the pubertal or adult PCOS mouse models (data not shown). Finally, the pubertal PCOS mouse model displayed signs of insulin resistance compared to placebo-treated mice while the adult PCOS mouse model remained insulin sensitive (Fig. 3c).

Letrozole treatment of adult female mice was not associated with a strong correlation between alpha diversity and time

Gut microbial diversity profiles were generated from 84 fecal samples taken prior to and during 5 weeks of placebo or letrozole treatment (weeks 0–5). Sequences collected before placebo and letrozole treatment were compared for both the pubertal and adult mouse models. No significant difference in alpha and beta diversity was observed between the two treatment groups at time 0, indicating that the gut microbiomes of the groups were similar prior to treatment for both the pubertal and adult cohort (Additional file 1: Figure S1). Similar to a previous study in pubertal mice [54], placebo-treated adult mice showed a strong positive correlation between alpha diversity and time as measured by species richness and phylogenetic diversity but not evenness of their gut communities (Fig. 4a, c, e). In contrast, letrozole treatment of adult mice was associated with a relatively weak positive correlation between alpha diversity and time (Fig. 3b, d, f). To examine this further, we evaluated whether there was a significant difference amongst the time points using a repeated measures (RM) ANOVA. RM-ANOVA found a highly significant effect of time on species richness and phylogenetic diversity in placebo-treated mice but no difference in letrozole-treated mice.

Letrozole treatment of adult female mice resulted in changes in gut microbiome beta diversity

UniFrac analyses were used to compare the similarity amongst gut microbial communities (beta diversity) in fecal samples from placebo versus letrozole–treated adult female mice. When all post-treatment data points were combined together, clustering of the data based on treatment was observed with unweighted UniFrac (Fig. 5a). When the samples were separated by the individual time points (Fig. 5b-f), Analysis of Similarity (ANOSIM) tests found a difference in the overall bacterial community composition of the gut microbiome between placebo and letrozole–treated adult female mice at weeks 4 and 5 post-treatment ($p = 0.01$ and $p = 0.03$ respectively). We also observed similar results using weighted UniFrac (data not shown).

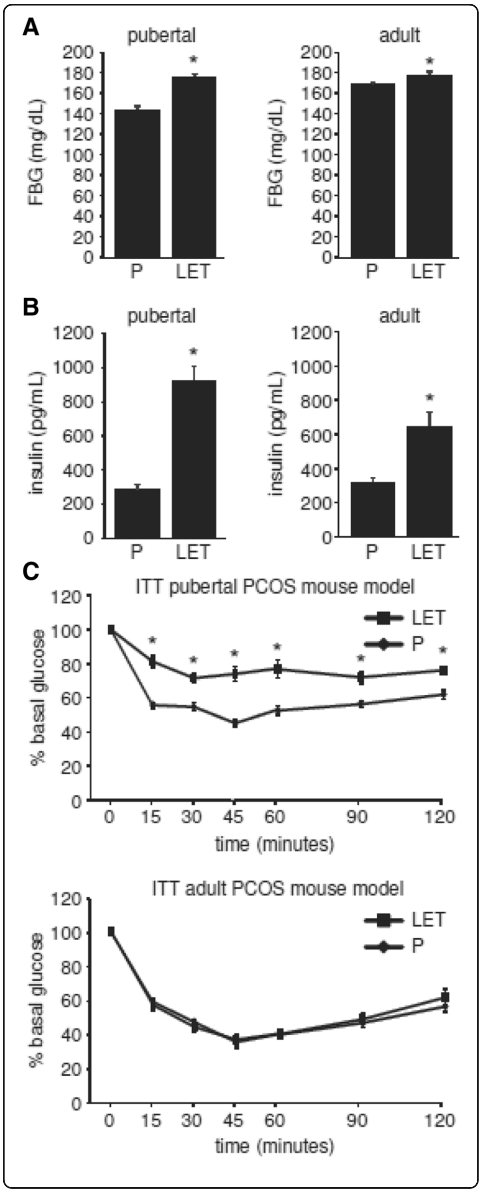

Fig. 3 Five weeks of letrozole treatment of adult female mice resulted in a minimal increase in fasting blood glucose and insulin levels and did not result in insulin resistance. The metabolic phenotype of pubertal (4 week-old) versus adult (8 week-old) female mice treated with placebo (P) or letrozole (LET) for 5 weeks was compared. LET treatment of adult female mice resulted in reduced fasting blood glucose (FBG) or insulin levels compared to pubertal mice (**a**-**b**). Unlike pubertal female mice, LET treatment of adult female mice for five weeks did not result in insulin resistance (**c**). Pubertal PCOS model ($n = 24$ P, $n = 22$ LET; adult PCOS model ($n = 8$ P, $n = 8$ LET). Student *t*-test or two-way repeated-measures ANOVA with post-hoc Student *t*-tests to directly compare P versus LET at specific time points were performed; * $p < 0.05$

Distinct bacterial genera discriminated between placebo and letrozole treatment in the pubertal and adult PCOS mouse models

In addition to studying changes in alpha and beta diversity, we also investigated whether the age at which letrozole treatment was initiated was important for changes in the taxonomic composition of the gut microbiome. We combined the post-treatment data (weeks 1–5) from placebo and letrozole-treated mice in the pubertal and adult mouse models. Based on the Greengenes taxonomic database, we identified a total of 10 bacterial phyla and 51 bacterial genera in the four different groups. Similar to our previous study [54], the majority of Operational Taxonomic Units (OTUs) in the adult mouse fecal samples were identified as Bacteriodetes or Firmicutes (~ 84–95%). We used RM-ANOVA to determine if the mean relative abundances of specific bacterial genera were different in the gut microbiome of placebo versus letrozole-treated mice in the pubertal and adult mouse models. A heatmap was generated to represent the relative abundance of 9 different bacterial genera that changed significantly with letrozole treatment (FDR-corrected $p < 0.05$) in the pubertal mouse model (Fig. 6a). Letrozole treatment of pubertal female mice resulted in higher relative abundances of *Coprococcus*, *Allobaculum*, *Bifidobacterium*, and an undescribed genus belonging to the Ruminococcaceae, as well as a lower abundance of *AF12*, *Dehalobacterium*, taxa belonging to the uncultured order YS2, and undescribed genera of Peptococcaceae and Bacteroidales (Fig. 6a).

In contrast to the pubertal mice, letrozole treatment of adult female mice resulted in changes in the mean relative abundance of a distinct set of 8 bacterial genera (FDR-corrected $p < 0.05$). With the exception of uncultured members of the genus-level *CF231* group, the rest of the genera from the Bacteroidetes phylum increased with letrozole treatment in

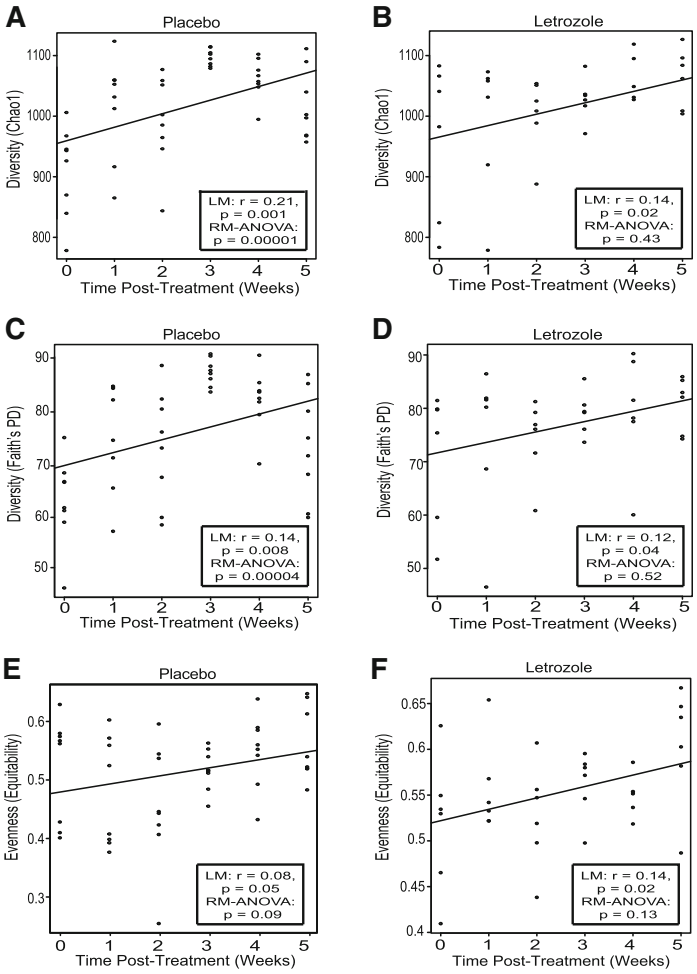

Fig. 4 Letrozole treatment of adult female mice did not result in a strong correlation between time and alpha diversity of the gut microbial community. Chao 1 species richness estimate per sample at each collection time for placebo (**a**) and letrozole-treated adult female mice (**b**), (n = 8 placebo, *n* = 6 letrozole). Faith's phylogenetic diversity (PD) estimate per sample at each collection time for placebo (**c**) and letrozole-treated adult female mice (**d**). Equitability (evenness) estimate per sample at each collection time point for placebo (**e**) and letrozole-treated mice (**f**). Line of best fit along with results of linear regression (LM) and repeated measures (RM) ANOVA are shown in box inset

adult female mice, including *Prevotella,* an uncultured genus within Parabacteroides and a genus-level group within the S24–7 family (Fig. 6c). Letrozole treatment of adult mice also resulted in a higher relative abundance of genera from Lachnospiraceae, Ruminococcaceae, and Peptococcaceae, as well as a lower abundance of *Lactobacillus* (Fig. 6c).

Random Forest classifier identified bacterial genera predictive of placebo and letrozole treatment in the pubertal and adult PCOS mouse models

The Random Forest (RF) classifier was trained to determine how well placebo or letrozole treatment could be predicted based on bacterial relative abundances in the two models (pubertal and adult). Forty-five of the 51

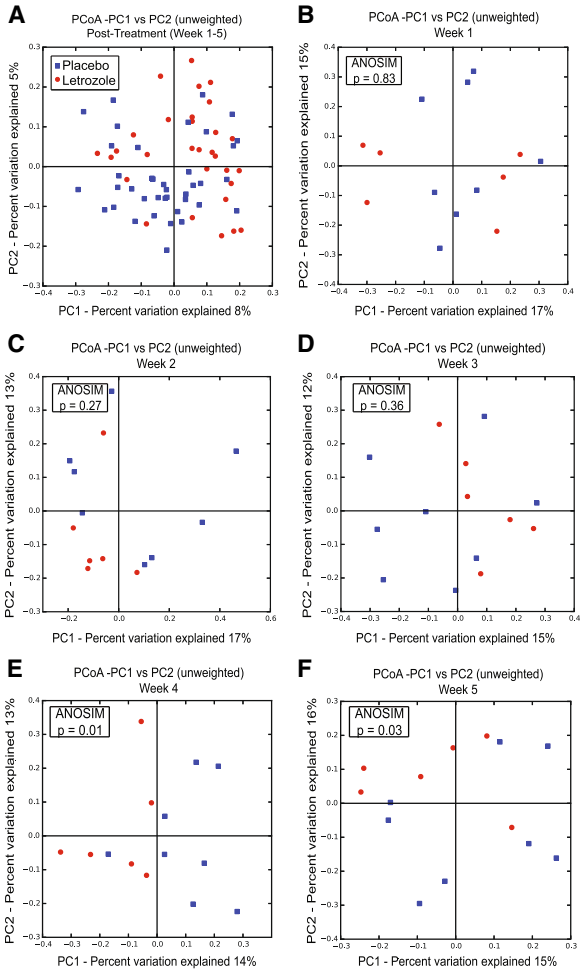

Fig. 5 Letrozole treatment of adult female mice resulted in a significant shift in the beta diversity of the gut microbiome. Principal Coordinates Analysis (PCoA) of unweighted UniFrac for samples collected post-treatment (weeks 1–5) were compared between placebo (n = 8) and letrozole-treated (n = 6) mice (**a**). Proportion of variance explained by each principal coordinate axis is denoted in the corresponding axis label. Samples from placebo- and letrozole-treated mice were then compared for each time point (**b-f**). Results of Analysis of Similarity (ANOSIM) tests are shown in the box inset

total bacterial genera identified in the four different groups were used for RF classification (six were excluded due to low relative abundances). Our results showed that RF predicted treatment category in the pubertal group with 78.5% accuracy while it predicted treatment category in the adult group with 84% accuracy (Table 1). Variable importance by mean decrease in accuracy was calculated for the RF models. Figure 6b and d illustrate 10 bacterial genera whose removal caused the greatest decrease in model accuracy (i.e. the most important for classification) in the pubertal and adult mouse models respectively. In the pubertal model, the removal of *Coprococcus*, *Allobaculum*, *AF12*, *Mucispirillum*, *Roseburia*, *Sutterella*, and an unknown genus from Bacteroidales had the greatest impact on classification (mean decrease accuracy > 8; Fig. 6b). In the adult mice, the

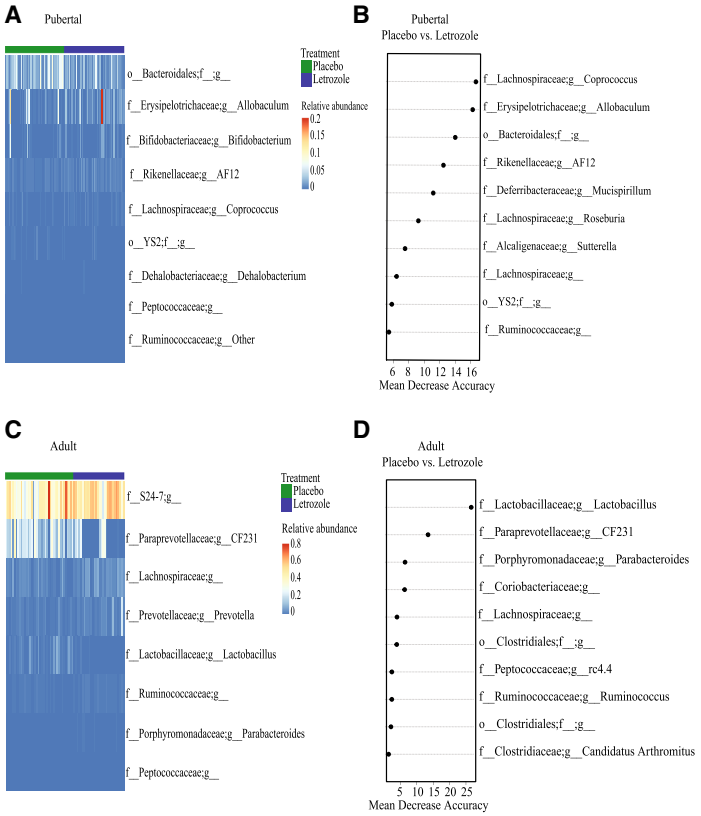

Fig. 6 Repeated measures analysis of variance and Random Forest classification identified distinct bacteria associated with letrozole treatment in the pubertal versus adult PCOS mouse model. Repeated measures analysis of variance (corrected for multiple comparisons via FDR) was used to determine whether the abundance of specific bacterial genus differed in placebo versus letrozole-treated pubertal or adult mice. A heatmap was generated for the bacterial genera that had a FDR adjusted *p*-value of < 0.05 and mean taxa abundance above 0.001 (**a, c**). The Random Forest classifier was used to identify bacterial genera that distinguished between placebo and letrozole treatment in pubertal or adult female mice (**b, d**). The top ten most discriminant bacterial genera in the models were displayed in the variable importance plots using a total of forty-five genera in the analysis. An increase in mean decrease accuracy reflects the prediction strength of the variable in classifying the different treatment groups

removal of *Lactobacillus*, *CF231* and *Parabacteriodes* caused the greatest decrease in prediction accuracy (mean decrease accuracy > 8; Fig. 6d).

Discussion

Our study demonstrated that initiation of letrozole treatment during puberty or adulthood in female mice resulted in reproductive hallmarks of PCOS, including elevated testosterone levels, anovulation and ovaries with cystic follicles. This suggests that the timing of androgen exposure (puberty versus adulthood) may not be important for development of the PCOS reproductive phenotype. On the other hand, our study did find a clear divergence between the metabolic phenotypes of the pubertal and adult mouse models. Similar to previously published studies [53, 54], letrozole treatment in pubertal female mice resulted in multiple metabolic features of PCOS, including obesity, abdominal adiposity, hyperinsulinemia, and insulin resistance. On the other hand, letrozole treatment in adult female mice did not result in substantial weight gain, abdominal adiposity or insulin resistance, indicating

Table 1 Classification error rates carried out using Random Forest classifiers composed of 500 trees

	Predicted classes		Classification error rates	OOB estimate of error rate	Accuracy
	Placebo	Letrozole			
Pubertal				21.5%	78.5%
Placebo	32	7	0.18		
Letrozole	10	30	0.25		
Adult				16.0%	84.0%
Placebo	30	1	0.03		
Letrozole	7	13	0.35		

that androgen exposure is not sufficient to induce the full PCOS-like metabolic phenotype in adult female mice. Interestingly, studies using post-natal treatment with DHT to create a hyperandrogenic mouse model observed a similar pattern: the metabolic phenotype depended on when DHT treatment was initiated. Compared with placebo-treated mice, female mice treated with DHT starting at 3 weeks of age gained significantly more weight, had greater levels of abdominal adiposity and were glucose intolerant [43, 47]. In contrast, while female mice treated with DHT in adulthood had impaired glucose tolerance, they did not become obese or display increased abdominal adiposity [56].

Our results also suggest that the timing of excess androgen exposure may be an important component in the development of the PCOS metabolic phenotype. Since PCOS often manifests in the early reproductive years, puberty has been suggested to be a critical developmental time period for the development and pathology of PCOS [6, 57]. Indeed, PCOS has been hypothesized to originate from abnormal pubertal development due to a lack of transition from an androgen-dominated state in early puberty to an estrogenic state in late puberty [58, 59]. Puberty is a time of considerable hormonal and metabolic change, including an increase in insulin resistance [60]. Although physiological insulin resistance is common in healthy adolescents, it usually resolves to prepubertal levels in adulthood [61]. Pubertal insulin resistance has been reported to increase the risk of developing type 2 diabetes along with accelerating the complications of diabetes [62–65]. Thus, it is possible that insulin resistance and the hyperinsulinemia that occurs during puberty may also contribute to the risk of developing obesity and metabolic dysfunction in PCOS.

Another factor that changes during the transition from childhood to adulthood is the gut microbiome. Studies have shown that children or adolescents have a distinct gut microbial community compared to adults [66, 67]. Moreover, prepubertal mice were reported to have a different gut microbiome than adult mice [68, 69]. Studies in humans and mice have shown a strong positive association between gut bacterial alpha diversity and age, indicating that the complexity of the gut microbiome increases as the host ages [70–72]. In contrast to placebo, there was no significant effect of time on alpha diversity in letrozole-treated mice when the data was adjusted for within subject error using RM-ANOVA (Fig. 4) [54]. With regards to beta diversity, letrozole treatment of both pubertal and adult female mice resulted in a distinct shift in the gut microbial composition (Fig. 5). However, closer examination of the types of bacteria that changed after letrozole treatment showed that the taxa driving the shift in beta diversity were quite distinct in the two mouse models (Fig. 6).

Letrozole treatment initiated during puberty resulted in changes in the abundances of bacterial genera previously reported to be altered in diet-induced obesity mouse models. In the pubertal model, RF and statistical analysis of relative bacterial abundances determined that *Coprococcus*, *Allobaculum* and an unknown genus from Bacteroidales differentiated the gut microbiomes of placebo and letrozole-treated mice (Fig. 6a and b). Significant differences were also observed in the relative abundance of *Bifidobacterium*, reported to have strain-specific effects on weight gain in rodents [73], as well as *Dehalobacterium* and unknown genera belonging to the Rikenellaceae and Ruminococcaceae families, all of which have been associated with obesity [28, 74–77]. The genus with the strongest effect on RF classification, namely *Coprococcus*, was previously reported to be more abundant in obese individuals [78, 79], in agreement with the higher levels observed after letrozole treatment in pubertal mice. The second most important genus in terms of classification, *Allobaculum*, was reported to be lower in the gut of obese mice fed a high-fat diet [80, 81], in contrast to the increase in *Allobaculum* observed after letrozole treatment.

In comparison to pubertal mice, letrozole treatment of adult female mice had a distinct impact on the composition of the gut microbial community. With the exception of a genus within the Peptococcaceae, the bacterial genera most affected by letrozole treatment in adult females were not altered in pubertal mice and vice versa (Fig. 6a, c). It should be noted that the genera that changed in the pubertal and adult female mice after letrozole treatment were present at both ages, indicating that the differential effects of letrozole treatment in the two models was not due to the absence of specific bacteria. The most striking difference in letrozole treatment of adult female mice was the importance that *Lactobacillus*, *Parabacteroides* and the uncultured Paraprevotellaceae group *CF231* played in classifying the treatment groups (Fig. 6d). The mean relative abundance of these bacteria changed significantly after letrozole treatment in adult

female mice (Fig. 6c). This is in contrast to the increased abundance of some *Lactobacillus* species observed in obese humans [82–84], though direct comparisons are difficult since there may be strain-specific effects of *Lactobacillus* on weight gain [85]. While *CF231* has not been described in much detail, members of the Paraprevotellaceae are found in the gut of many mammals [86, 87] and have been suggested to be involved in the degradation of plant polysaccharides into short chain fatty acids [88]. *Parabacteroides* are also known to metabolize non-digestible carbohydrates, but the increase in *Parabacteroides* relative abundance after 5 weeks of letrozole treatment contrasts with the decrease observed in mice fed a high-fat diet [27, 89].

Conclusions

In summary, our study demonstrated that the timing of androgen exposure may be important for development of the PCOS metabolic phenotype and associated changes in the gut microbiome. While letrozole treatment of female mice during puberty and in adulthood both resulted in reproductive hallmarks of PCOS, including hyperandrogenemia, anovulation and polycystic ovaries, letrozole treatment in adulthood did not result in the weight gain, abdominal adiposity or insulin resistance observed in the pubertal PCOS mouse model. In addition, letrozole treatment in adulthood resulted in distinct changes in the gut microbiome, particularly in *Lactobacillus*. Although evidence is accumulating that changes in steroid hormones are associated with an altered gut microbiome [90], the mechanisms involved in steroid hormone/gut microbe interactions are currently unknown. Future studies investigating whether steroid hormones regulate the gut microbiome through actions in the gastrointestinal tract, immune system or other tissues will begin to address the mechanisms involved.

Given that many of the previous studies that report an association of specific bacterial genera with obesity in humans and high fat diet-induced mouse models are contradictory, it is possible that these results are due to modulation of specific bacterial species and strains within genera. Future studies should employ higher resolution methods such as metagenomic sequencing or quantitative PCR to fully understand the effects of hyperandrogenism on the gut microbiome. Moreover, since many studies of the role of the gut microbiome in obesity are confounded by the effect of diet on the microbiome, the letrozole-induced PCOS mouse model provides an opportunity to study the effects of androgen excess on the gut microbiome and metabolism in a diet-independent setting, since food intake is not altered by letrozole treatment [55]. Moreover, the adult PCOS mouse model can be used to study the effects of hyperandrogenism in female mice without the confounding variable of insulin resistance. Further studies addressing whether the gut microbiome plays a causal role in the development of PCOS or if manipulation of the gut microbiome can improve the PCOS phenotype will be informative. In addition, prospective studies with adolescent girls may be crucial to understand the etiology and development of PCOS, particularly the metabolic dysregulation and changes in the gut microbiome associated with this disease.

Methods

PCOS mouse model

C57BL/6NHsd female mice purchased from Envigo were housed in a vivarium for one week under specific pathogen-free conditions with an automatic *12 h:12 h light/dark* cycle (*light* period: 06.00–18.00) and ad libitum access to water and food (Teklad Global 18% Protein Extruded Diet, Envigo). Prior to the beginning of the study, the mice were sorted by weight to ensure that the starting weight was similar between the two treatment groups. To establish the pubertal or adult PCOS models, 4 or 8 week-old female mice, respectively were implanted subcutaneously with a placebo or 3 mg letrozole pellet (3 mm diameter; Innovative Research of America) that provided a slow, constant release of letrozole (50 μg/day) over 5 weeks. For the duration of the experiment, the mice were housed 2 per cage: 2 placebo or 2 letrozole-treated mice. Placebo and letrozole-treated mice were not housed together to avoid the influence of coprophagy on the PCOS mouse model. At the end of the study, the mice were sacrificed using 2.5% isoflurane delivered with a precision vaporizer followed by a physical method of euthanasia.

Analysis of reproductive and metabolic phenotype

The mice were weighed weekly. The stage of the estrous cycle for placebo and letrozole-treated mice was determined from the predominant cell type in vaginal epithelium smears obtained during weeks 4–5 of treatment. After 5 weeks of placebo or letrozole treatment, the mice were fasted for 5 h and blood from the tail vein was collected to measure fasting insulin levels. Blood glucose was measured using a handheld glucometer (One Touch UltraMini, LifeScan, Inc) and an intraperitoneal (IP) insulin tolerance test (ITT) was performed. Tail vein blood glucose was measured just before (time 0) an IP injection of insulin (0.75 U/kg in sterile saline; Humulin R U-100, Eli Lilly) was given and at 15, 30, 45, 60, 90, and 120 min post injection.

At the end of the experiment, blood was collected from the posterior vena cava, parametrial fat pads were dissected and weighed, and the ovaries were dissected, weighed, fixed in 4% paraformaldehyde at 4 °C overnight,

and stored in 70% ethanol before processing for histology. Paraffin-embedded ovaries were sectioned at 10 μm and stained with hematoxylin and eosin (Zyagen). Serum testosterone were measured using a mouse ELISA (range 10–800 ng/dL) while LH levels were measured using a radioimmunoassay (range 0.04–75 ng/mL) by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility. Serum insulin was measured using a mouse ELISA (ALPO) by the University of California, Davis Mouse Metabolic Phenotyping Center. The data from four mice in the pubertal and adult letrozole-treated groups were removed from the analyses because these mice did not have a significant elevation in serum testosterone when compared to the average of the placebo-treated mice. The analysis of the reproductive and metabolic phenotypes was performed with data from 2 unpublished cohorts of the adult PCOS model (total $n = 16$ placebo, $n = 14$ letrozole) and 3 cohorts of the pubertal PCOS model (total $n = 24$ placebo, $n = 22$ letrozole) including 2 unpublished and 1 previously published cohort [54]. Differences between placebo and letrozole treatment were analyzed by Student *t*-test or two-way repeated measures ANOVA followed by post-hoc comparisons of individual time points.

Fecal sample collection and DNA isolation

Fecal samples were collected from one cohort of 8-week-old female mice ($n = 8$/group) prior to treatment with placebo or letrozole and once per week thereafter for 5 weeks. Fecal samples were frozen immediately after collection and stored at − 80 °C. Bacterial DNA was extracted from the fecal samples using the MoBio PowerSoil DNA Extraction Kit following the manufacturer's protocol, and the DNA was stored at − 80 °C.

16S rRNA amplicon sequencing

The V4 hypervariable region of the 16S rRNA gene was PCR amplified with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) [91]. The reverse primers contained unique 12 base pair Golay barcodes that were incorporated into the PCR amplicons [92]. The barcoded primers allowed for pooling of multiple PCR amplicons in a single sequencing run. Thermocycling parameters were as follows: denaturing at 98 °C for 2 min followed by amplification for 35 cycles at 98 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s, and a final extension of 72 °C for 10 min. The resulting amplicons were submitted to The Scripps Research Institute Next Generation Sequencing Core Facility where they were cleaned using Zymo DNA Clean & Concentrator™-25 columns, quantified using a Qubit Flourometer (Life Technologies) and pooled. Pooled PCR products were size selected on a 2% agarose gel (290–350 bp), purified using a Zymoclean™ Gel DNA recovery kit and used to prepare sequencing libraries following the recommended Illumina protocol involving end-repair, A-tailing and adapter ligation. The DNA library was then size selected on a 2% agarose gel (410–470 bp), cleaned using the Agencourt SPRI system (Beckman Coulter, Inc.) and PCR amplified with HiFi Polymerase (Kapa Biosystems) for 12 cycles. The amplified DNA products were again size selected on a 2% agarose gel and purified using the Zymoclean™ Gel DNA recovery kit. The purified DNA library was quantitated, denatured in 0.1 N NaOH and diluted to a final concentration of 5 pM before being loaded onto the Illumina single read flow-cell for sequencing on the Illumina MiSeq system along with 4 pM PhiX control library.

16S rRNA gene sequence analysis

16S rRNA sequences for the adult mice were de-multiplexed using the Quantitative Insights Into Microbial Ecology (QIIME v.1.9.1, http://www.qiime.org) pipeline [93] using the default split_libraries.py script parameter [94]. This resulted in approximately 4.3 million Illumina sequences across all samples with an average of 50,000 sequences per sample. Sequences from two mice in the letrozole-treated group were removed from the analysis because these mice did not have a significant elevation in serum testosterone levels compared to the average of the placebo-treated mice. The 16S rRNA gene sequencing quality control and analysis for the samples from the adult mice followed the same pipeline as the samples in a previously published study with placebo or letrozole-treated pubertal female mice [54]. Sequences were clustered using the pick_de_novo_otus.py script with usearch [95]. Sequences were assigned to OTUs with an assumed 97% threshold of pairwise identity for bacterial species by comparison with the Greengenes reference database [96] using the RDP classifier [97]. Before performing downstream analysis, singletons and OTUs present in less than 25% of the samples were discarded from the database to minimize the effect of spurious, low abundance sequences using the filter_otus_from_otu_table.py script. Sequences were then aligned using PyNast [93] and a phylogenetic tree constructed using FastTree [98]. The alpha_diversity.py script was used to estimate several different attributes of alpha diversity. Species richness was estimated using Chao1 to define the total number of unique species in a community [99]. Faith's Phylogenetic Diversity was used to measure the phylogenetic diversity of a community by calculating the total branch lengths on a phylogenetic tree of all members of the community [100]. Evenness was estimated using the Equitability index [101]. The beta_diversity_through_plots.py script was used to compute weighted and unweighted UniFrac distances [102]. The smaller the UniFrac distance between two microbial

communities, the more similar the communities are in their overall diversity. The weighted UniFrac distance metric incorporates the abundance of specific taxa in each community into the UniFrac distance calculation while unweighted UniFrac ignores abundance information. Taxonomic distributions across sample categories were calculated (from phylum to genus) using the summarize_taxa_through_plots.py script. Several bacterial genera such as *Anaeroplasma* and an unknown Enterobacteriaceae were excluded from the analysis because of extremely low abundance, suggesting that they may have been artifacts. Sequences from placebo-treated samples collected during week 5 (9 weeks of age) of the pubertal cohort were compared to samples collected from week 1 (9 weeks of age) from the adult cohort. No significant difference in alpha and beta diversity was observed between the two cohorts, indicating that the gut microbiome at the end of placebo treatment in the pubertal cohort was similar to the gut microbiome at the beginning of placebo treatment in the adult cohort.

Statistical analysis

Pearson's product-moment correlation was performed when analyzing alpha diversity over time using the RStudio statistical package (version 0.99.893). RM-ANOVA was used to model alpha diversity measures accounting for within subject error. Two-dimensional PCoA plots were constructed using the make_2d_plots.py script in QIIME. ANOSIM tests for weighted and unweighted UniFrac distances between treatments were performed using the compare_categories.py script. The biom table of post treatment samples (weeks 1–5) from the adult study was merged with the biom table from the pubertal study, resulting in approximately 6.2 million sequences from 170 samples (pubertal = 100 samples; adult = 70 samples). The merged dataset was used to compare differences among treatment group and developmental stage. RM-ANOVA adjusting for within subject error (corrected for multiple comparisons via FDR) was used to determine whether the abundance of specific bacterial genus differed between treatments. The RF supervised machine-learning classifier was used to determine how well a given set of factors (e.g. bacterial genera) classified discrete categories and which factors were most important for the classification [70, 116]. RF was implemented in R using the "randomForest" library, and was used to identify bacterial genera that differentiated placebo and letrozole treatment within pubertal or adult mice.

Additional file

Additional file 1: Figure S1. No differences in gut microbial community diversity between placebo and letrozole-treated mice were observed prior to treatment. No significant differences in gut microbiome alpha diversity (Faith's PD) between placebo- and letrozole-treated mice were observed prior to treatment (week 0) in the pubertal (placebo n = 10, letrozole n = 10) (A) or adult (placebo n = 8, letrozole n = 6) (B) model. Similarly, no differences in beta diversity (unweighted UniFrac) were observed between placebo- and letrozole-treated mice prior to treatment in the pubertal (C) or adult model (D). Student t-test was used to compare alpha diversity between groups and Analysis of Similarity (ANOSIM) test was used to compare beta diversity between groups. (PDF 1335 kb)

Abbreviations

ANOSIM: Analysis of similarity; FBG: Fasting blood glucose; IP: Intraperitoneal; ITT: Insulin tolerance test; OTU: Operational taxonomic unit; PCOS: Polycystic ovary syndrome; QIIME: Quantitative Insights Into Microbial Ecology; RF: Random Forest; RM-ANOVA: Repeated measures analysis of variance

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Availability of data and materials

The 16S rRNA sequence dataset generated and analyzed during the current study is available in the European Nucleotide Archive (Study Accession Number PRJEB20895).

Authors' contributions

VT and SK conceived and designed the study; PJT, DVS, BSH, LS and ARA performed reproductive and metabolic assessments, and collected samples; PJT performed DNA extractions and PCR amplifications; PJT, VT and SK analyzed the data; PT, SK and VT wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures in this study were approved by the University of California, San Diego Institutional Animal Care and Use Committee (protocol number S14011).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 3

Exposure to a Healthy Gut Microbiome Protects Against Reproductive and Metabolic Dysregulation in a PCOS Mouse Model

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Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting ~10% to 15% of reproductive-aged women worldwide. Diagnosis requires two of the following: hyperandrogenism, oligo-ovulation or anovulation, and polycystic ovaries. In addition to reproductive dysfunction, many women with PCOS display metabolic abnormalities associated with hyperandrogenism. Recent studies have reported that the gut microbiome is altered in women with PCOS and rodent models of the disorder. However, it is unknown whether the gut microbiome plays a causal role in the development and pathology of PCOS. Given its potential role, we hypothesized that exposure to a healthy gut microbiome would protect against development of PCOS. A cohousing study was performed using a letrozole-induced PCOS mouse model that recapitulates many reproductive and metabolic characteristics of PCOS. Because mice are coprophagic, cohousing results in repeated, noninvasive inoculation of gut microbes in cohoused mice via the fecal-oral route. In contrast to letrozole-treated mice housed together, letrozole mice cohoused with placebo mice showed significant improvement in both reproductive and metabolic PCOS phenotypes. Using 16S rRNA gene sequencing, we also observed that the overall composition of the gut microbiome and the relative abundance of *Coprobacillus* and *Lactobacillus* differed in letrozole-treated mice cohoused with placebo mice compared with letrozole mice housed together. These results suggest that dysbiosis of the gut microbiome may play a causal role in PCOS and that modulation of the gut microbiome may be a potential treatment option for PCOS. (***Endocrinology*** **160: 1193–1204, 2019)**

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting ~10% to 15% of women worldwide (1). Diagnosis of PCOS, based on the Rotterdam Consensus criteria (2003), requires two of the following: hyperandrogenism, oligomenorrhea or amenorrhea, and polycystic ovaries. PCOS is the leading cause of anovulatory infertility in women, and women with PCOS also have an elevated likelihood of miscarriage and pregnancy complications (1–3). Although it is often perceived as a reproductive disorder, PCOS is also a metabolic disorder. Women with PCOS have an elevated risk of developing obesity, type 2 diabetes, hypertension, and nonalcoholic fatty liver disease (2, 4, 5). PCOS-related metabolic dysfunction is associated with hyperandrogenism and occurs irrespective of body mass index (6, 7). Although studies indicate that androgen excess is an important contributor to metabolic dysregulation in women with PCOS, the mechanisms that lead to obesity and insulin resistance in PCOS are not well understood. Although genetic and environmental factors undoubtedly influence the development and pathology of PCOS (6, 8–10), it is worth exploring whether gut microbes contribute to this disorder.

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Abbreviations: CAP, canonical analysis of principal coordinates; FBG, fasting blood glucose; LET, letrozole mice housed together; LET^{ch}, letrozole mice cohoused with placebo mice; P, placebo mice housed together; P^{ch}, placebo mice cohoused with letrozole mice; PCoA, principal coordinate analysis; PCOS, polycystic ovary syndrome; PD, phylogenetic diversity; PERMANOVA, permutational multivariate analysis of variance; SV, sequence variant.

Studies over the past decade have shown that the gastrointestinal tract harbors a complex microbial ecosystem (the gut microbiome) that is important for human health and disease (11, 12). Gut microbes offer many benefits to the host, including protection against pathogens and regulation of host immunity and the integrity of the intestinal barrier (13–15). The gut microbiome is also involved in the production of short-chain fatty acids via fermentation of dietary fibers, production of essential vitamins such as folic acid and B12, and modification of bile acids, neurotransmitters, and hormones (16, 17). Studies have also shown that changes in the gut microbiome are associated with metabolic disorders such as obesity and type 2 diabetes (18, 19). Moreover, studies have reported that transplantation of stool from obese donors into germ-free mice results in an obese phenotype (20), suggesting that the gut microbiome may play a causative role in metabolic dysregulation. These transplantation studies were complemented with cohousing studies that took advantage of the fact that, because mice are coprophagic, cohousing provides a means for repeated, noninvasive microbial inoculation. Cohousing germ-free mice transplanted with stool from obese donors with germ-free mice transplanted with stool from lean donors was shown to protect the mice transplanted with obese donor stool from developing obesity (20–22). Altogether, these studies suggest that modulation of the gut microbiome may be a potential treatment option for metabolic disorders.

With regard to PCOS, several recent studies reported that changes in the gut microbiome are associated with PCOS (23–26). These studies detected lower α-diversity and differences in the relative abundances of specific Bacteroidetes and Firmicutes in women with PCOS compared with controls (23–25). In particular, changes in the relative abundance of bacterial genera from the Bacteroidaceae, Clostridaceae, Erysipelotrichidae, Lachnospiraceae, Lactobacillaceae, Porphyromondaceae, Ruminococcaceae, and S24-7 families were observed in several studies. In addition, changes in the gut microbiome correlated with hyperandrogenism (23–25), suggesting that testosterone may influence the composition of the gut microbiome in women. In addition to studies in humans, several studies reported a significant association between the gut microbiome and PCOS in rodent models (27–30). Because the rodent models are diet independent, these studies suggest that the mechanisms that result in an altered gut microbiome in PCOS are distinct from diet-induced effects on the gut microbiome observed in high-fat diet–induced obesity models. Overall, these studies indicate that the gut microbiome of women with PCOS differs significantly from that of healthy women and suggest that a microbial imbalance or "dysbiosis" in the gut may contribute to the pathology of PCOS.

We previously developed a PCOS mouse model that uses treatment with letrozole, a nonsteroidal aromatase inhibitor, to increase testosterone levels and decrease estrogen levels by inhibiting the conversion of testosterone to estrogen (31). Letrozole treatment of pubertal female mice results in reproductive hallmarks of PCOS including hyperandrogenism, acyclicity, polycystic ovaries, and elevated LH levels (31). This model also exhibits metabolic dysregulation similar to the phenotype in women with PCOS, including weight gain, abdominal adiposity, elevated fasting blood glucose (FBG) and insulin levels, impaired glucose-stimulated insulin secretion, insulin resistance, and dyslipidemia (32). Our studies also showed that letrozole treatment did not alter food intake or energy expenditure, even though locomotion was decreased (32), suggesting that other mechanisms contribute to the metabolic dysregulation in this model. Although letrozole treatment results in estrogen levels that are lower than estrogen levels in women with PCOS, we used this model to study the role of the gut microbiome in PCOS because it recapitulates both reproductive and metabolic aspects of PCOS. As in women with PCOS, 16S rRNA gene sequencing showed that letrozole treatment was associated with lower gut microbial richness, a shift in the overall gut microbial composition, and changes in specific Bacteroidetes and Firmicutes (27). A recent study examining the effects of nonantibiotic drugs on the gut microbiome found that letrozole did not alter growth of ~40 representative gut bacteria (33), which suggests that differences in the gut microbial composition found in the PCOS mouse model are not a direct effect of letrozole.

To begin to address whether the gut microbiome contributes to the pathophysiology of PCOS and whether manipulation of the gut microbiome can be used to treat PCOS, we used a cohousing paradigm to determine whether exposure to a healthy gut microbiome protected against development of PCOS metabolic and reproductive phenotypes. Because mice are coprophagic, gut microbes can be readily transferred from one mouse to another through the fecal-oral route. In this study, pubertal female mice were treated with placebo or letrozole and housed two per cage in three different housing arrangements. The study groups consisted of placebo mice housed together (P), letrozole mice housed together (LET), placebo mice cohoused with letrozole mice (P^{ch}), and letrozole mice cohoused with placebo mice (LET^{ch}). Overall, cohousing letrozole with placebo mice resulted in substantial improvement in both PCOS metabolic and reproductive phenotypes compared with letrozole mice housed together. Furthermore, 16S rRNA gene sequence analysis demonstrated that cohousing letrozole with placebo mice resulted in changes in the

β-diversity of the gut microbiome and highlighted bacteria that may be candidates for probiotic therapy. Our findings support the idea that there may be a causal link between the gut microbiome and PCOS and that modulation of the gut microbiome may be a potential treatment option for PCOS.

Materials and Methods

PCOS mouse model

C57BL/6NHsd female mice from Envigo were housed in a vivarium under specific pathogen-free conditions with an automatic 12 hour:12 hour light/dark cycle (light period: 06:00 to 18:00) and *ad libitum* access to water and food (Teklad S-2335 Mouse Breeder Irradiated Diet, Envigo). To establish the pubertal PCOS model, 4-week-old female mice were implanted subcutaneously with a placebo or 3-mg letrozole pellet (3 mm diameter; 50 μg/d; Innovative Research of America) for 5 weeks. The 50 μg/d dose was based on the original letrozole mouse model study (31). For the cohousing paradigm, mice were housed two per cage in three different cage arrangements: two placebo mice, two letrozole mice, or one placebo and one letrozole mouse. The cohousing experimental design resulted in four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch}. All animal procedures in this study were approved by the University of California, San Diego Institutional Animal Care and Use Committee (Protocol Number S14011).

Analysis of reproductive phenotype

Mice were weighed weekly. Estrous cycle stage was determined from the predominant cell type in vaginal epithelial smears obtained during weeks 4 to 5 of treatment as previously described (31). At the end of the experiment, ovaries were dissected, weighed, fixed in 4% paraformaldehyde, paraffin-embedded, sectioned at 10 μm, and stained with hematoxylin and eosin (Zyagen). Serum testosterone and LH levels were measured using a mouse ELISA (34) (range 10 to 800 ng/dL) and a radioimmunoassay (35, 36) (range 0.04 to 75 ng/mL), by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility.

Analysis of metabolic phenotype

After 5 weeks of treatment, mice were fasted for 5 hours and blood from the tail vein was collected to measure fasting insulin levels. Blood glucose was measured with a handheld glucometer (One Touch UltraMini, LifeScan, Inc), and an intraperitoneal insulin tolerance test was performed. Tail vein blood glucose was measured just before (time 0) an intraperitoneal injection of insulin (0.75 U/kg in sterile saline; Humulin R U-100, Eli Lilly) was given and at 15, 30, 45, 60, 90, and 120 minutes after injection. At the end of the experiment, the mice were anesthetized with isoflurane, blood was collected from the posterior vena cava, and parametrial fat pads were dissected and weighed. Serum insulin was measured with a mouse ELISA (37) by the University of California, Davis Mouse Metabolic Phenotyping Center.

Quantitative real-time PCR of ovarian genes

Total RNA was isolated from ovaries with an RNeasy Mini Plus kit (Qiagen), which also removes genomic DNA. cDNA was made by reverse transcription of total RNA with an iScript cDNA synthesis kit (Bio-Rad Laboratories). cDNA products were detected with SYBR Green Supermix (Bio-Rad Laboratories) on a Bio-Rad CFX Connect quantitative real-time PCR system (Bio-Rad Laboratories) with previously described primers (31). Data were analyzed by the $2^{-\Delta\Delta CT}$ method (38) by normalizing the gene of interest to glyceraldehyde 3-phosphate dehydrogenase. Data were represented as mean fold change compared with placebo ± the SEM.

Statistical analysis of reproductive and metabolic phenotypes

The statistical package JMP 13 (SAS) was used to analyze differences between groups by one-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test or two-way repeated-measures ANOVA followed by *post hoc* comparisons of individual time points. Different letters or an asterisk were used to indicate significant differences ($P < 0.05$).

Fecal sample collection, DNA isolation, and 16S rRNA gene sequencing

Fecal samples were collected from 8 mice per group (32 mice total) before treatment and once per week for 5 weeks. Fecal samples were frozen immediately after collection and stored at −80°C. Bacterial DNA was extracted from the samples with the DNeasy PowerSoil Kit (Qiagen) and stored at −80°C. The V4 hypervariable region of the 16S rRNA gene was PCR amplified with primers 515F and 806R (39). The reverse primers contained unique 12-bp Golay barcodes that were incorporated into the PCR amplicons (39). Amplicon sequence libraries were prepared at the Scripps Research Institute Next Generation Sequencing Core Facility, where the libraries were sequenced on an Illumina MiSeq as previously described (27).

16S rRNA gene sequence analysis

Raw sequences were imported into QIIME 2 (version 2018.4) with the q2-tools-import script, and sequences were demultiplexed with the q2-demux emp-single script. This procedure resulted in 7.3 million sequences, with an average of 36,000 sequences per sample. The 16S rRNA sequences generated in this study were deposited into the European Nucleotide Archive (Study Accession Number PRJEB29583). DADA2 software was used to obtain a set of observed sequence variants (SVs) (40). Based on the quality scores, the forward reads were truncated at position 240 with the q2-dada2-denoise script. Taxonomy was assigned with a pretrained naive Bayes classifier (Greengenes 13_8 99% operational taxonomic units) and the q2-feature-classifier plugin (41). Out of 192 samples, 5 were removed because of insufficient sequence coverage (one placebo at week 4, two P^{ch} at week 5, and two LET^{ch} at week 5), resulting in 187 samples. In total, 318 SVs were identified from 186 fecal samples. The resulting SVs were then aligned in MAFFT (42), and a phylogenetic tree was built in FastTree (43). Taxonomic distributions of the samples were calculated with the q2-taxa-barplot script. α- and β-diversity metrics were computed with the q2-diversity core-metrics script at a rarefied sampling depth of 1250. The α-diversity metric, Faith phylogenetic diversity (PD), was used to measure phylogenetic biodiversity by calculating the total branch lengths on a phylogenetic tree of all members in a community (44). UniFrac

was used to compare the similarity (β-diversity) between the microbial communities by calculating the shared PD between pairs of microbial communities (45, 46).

Statistical analysis of 16S rRNA sequences

Statistical calculations were performed in the R statistical package (version 3.5.1) with the phyloseq (version 1.26.1) (47) and vegan package (version 2.5.3). α-Diversity data were tested for normality via the Shapiro-Wilk test. Variables that were not normally distributed were ranked. Changes in α-diversity over time were analyzed via simple linear regression and Pearson rank correlation on ranked diversity measures. Linear mixed effects analysis of the relationship between α-diversity and time was done with the lme4 R package (version 1.1.19). *P* values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. Principal coordinate analysis (PCoA) and canonical analysis of principal coordinates (CAP) plots (48) were constructed in the phyloseq R package. PCoA plots were used to represent the similarity of posttreatment (weeks 1 to 5) fecal microbiome samples based on multiple variables in the data set, and CAP was used to visualize the relationship of the fecal microbiome with specific parameters. Permutational multivariate analysis of variance (PERMANOVA) used posttreatment weighted UniFrac distance measures to assess bacterial community compositional differences and its relationship to cohousing treatment group (999 permutations "vegan" package). DESeq2 (49) (version 1.14.1) in the microbiomeSeq package (version 0.1, http://www.github.com/umerijaz/microbiomeSeq) was used to identify bacterial genera that were differentially abundant between placebo and LET mice and between LET^{ch} and LET.

Results

Cohousing letrozole mice with placebo mice resulted in less weight gain and abdominal adiposity

To investigate whether exposure to a healthy gut microbiome can protect against the development of a PCOS metabolic and reproductive phenotype, we performed a cohousing study. Female mice were implanted with a placebo or letrozole pellet at 4 weeks of age and housed two mice per cage in three different housing arrangements. This study design resulted in four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch} (Fig. 1A). As shown in Fig. 1B, weight was measured each week during the 5 weeks of treatment. Similarly to previously published studies (27, 32), 2 weeks of letrozole treatment resulted in increased weight compared with placebo treatment that was maintained for the rest of the study (Fig. 1B). Five weeks of letrozole treatment also resulted in greater abdominal adiposity compared with placebo treatment (Fig. 1C). Interestingly, P^{ch} mice had similar weight gain and abdominal adiposity compared with placebo mice. In contrast, LET^{ch} mice gained less weight and exhibited a trend toward less abdominal adiposity compared with LET mice (Fig. 1B and 1C). Notably, the protective effect of cohousing letrozole mice with placebo mice on weight gain manifested only after several weeks of treatment.

Cohousing letrozole mice with placebo mice resulted in reduced FBG and insulin levels and insulin resistance

As reported in previous studies (27, 31, 32), 5 weeks of letrozole treatment resulted in increased FBG and insulin levels and insulin resistance (Fig. 1D–1F). P^{ch} mice had similar serum glucose and insulin levels and insulin sensitivity to that of placebo mice, whereas LET^{ch} mice had reduced FBG and insulin levels as well as less insulin resistance compared with LET mice (Fig. 1D–1F).

Cohousing letrozole mice with placebo mice resulted in estrous cyclicity

In addition to characterizing the effect of cohousing on the PCOS metabolic phenotype, we also assessed the effect on the reproductive axis. As previously published (27, 31), letrozole treatment resulted in hallmarks of PCOS, including elevated testosterone and LH levels and acyclicity in LET mice (Fig. 2A–2C). P^{ch} mice did not have changes in testosterone, LH, or estrous cyclicity compared with placebo mice (Fig. 2A–2C). On the other hand, LET^{ch} mice had decreased testosterone and LH levels compared with LET mice (Fig. 2A and 2B). In addition, LET^{ch} mice displayed changes in the morphology of vaginal epithelial cells representative of different stages of the estrous cycle compared with the constant diestrus exhibited by LET mice (Fig. 2C).

Cohousing letrozole mice with placebo mice protected ovarian function

Consistent with previous reports (27, 31), the ovaries of LET mice lacked corpora lutea and displayed cystic follicles and hemorrhagic cysts, whereas the ovaries of P^{ch} mice had a similar morphology to that of placebo mice (Fig. 3A). Interestingly, the ovaries of LET^{ch} mice lacked cystic follicles and hemorrhagic cysts and contained corpora lutea (Fig. 3A). As in previous reports (31), LET mice showed a significant increase in both ovarian weight and ovarian mRNA expression levels of follicle-stimulating hormone receptor (*Fshr*), cytochrome P450 17A1 (*Cyp17*), and aromatase (*Cyp19*) compared with placebo mice (Fig. 3B–3E). The ovarian weight and mRNA expression levels in P^{ch} mice mirrored those of placebo mice (Fig. 3B–3E). Compared with LET mice, LET^{ch} mice showed a significant decrease in ovarian weight and mRNA expression levels of *Cyp17*, whereas *Fshr* and *Cyp19* mRNA expression levels were comparable to those of LET mice (Fig. 3B–3E).

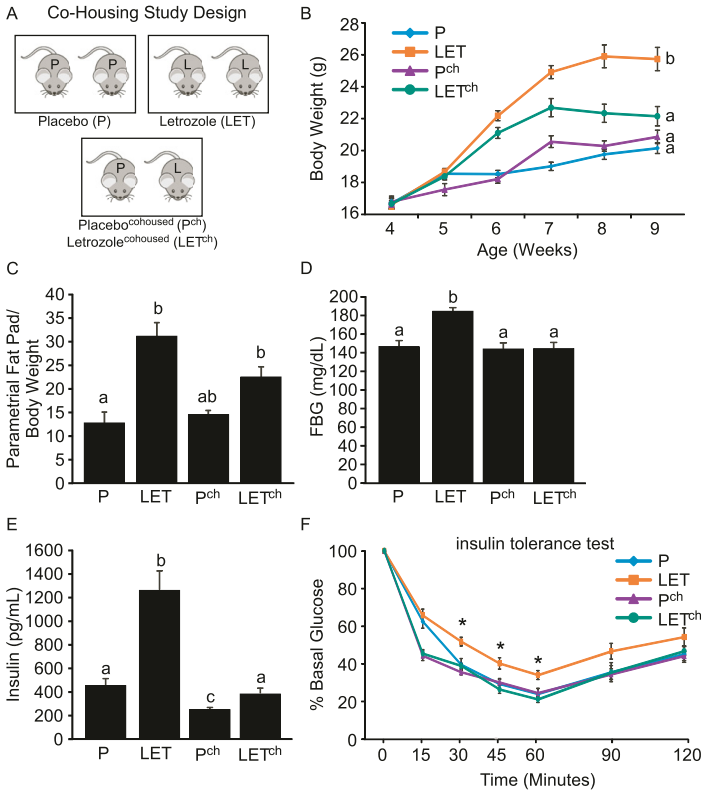

Figure 1. Cohousing letrozole mice with placebo mice protected against development of the PCOS metabolic phenotype. Design of cohousing study with pubertal female mice housed two per cage in three different housing arrangements that resulted in four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch} (A). Letrozole treatment resulted in metabolic dysfunction compared with placebo including (B–F) increased weight, abdominal adiposity, FBG, and insulin levels and insulin resistance. (B–F) Compared with LET mice, LET^{ch} mice showed a decrease in body weight, a decrease in abdominal adiposity, a decrease in FBG and insulin levels, and restored insulin sensitivity. Graph error bars represent SEM. Different letters or an asterisk symbol were used to indicate significant differences in a one-way ANOVA or repeated-measures two-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test ($P < 0.05$).

Gut microbial richness did not correlate with an improved PCOS phenotype

The overall composition of the gut microbiome from samples collected before placebo and letrozole treatment (time 0) was compared between the four groups. No significant difference in α- or β-diversity was observed between the groups, indicating that the gut microbiomes were similar before treatment (Fig. 4). Linear regression was used to examine the relationship between α-diversity of the gut microbiome (Faith PD) and time. There was a strong positive relationship between α-diversity and time in placebo mice ($r = 0.23$) but not LET mice ($r = 0.05$) (Fig. 5A and 5B). To account for the repeated measures in this longitudinal study, we also used a linear mixed-effect model to examine the association between microbial diversity and time. This analysis confirmed that there was a significant effect of time on α-diversity in placebo mice ($P = 0.003$) but not LET mice ($P = 0.2$) (Fig. 5A and 5B). We then investigated whether changes in α-diversity correlated with improved metabolic and reproductive phenotypes in the cohoused mice. In contrast to placebo mice, we did not observe a significant effect of time on α-diversity on linear regression or the linear mixed-effect model in P^{ch} mice ($r = 0.009$; $P = 1$) or LET^{ch} mice ($r = 0.08$; $P = 0.71$) (Fig. 5C and 5D).

Composition of the gut microbiome was altered by cohousing

In addition to investigating changes in α-diversity, we used weighted UniFrac distances to compare the similarity of gut microbial composition (β-diversity) between the different groups. Although visualization of the UniFrac distances via PCoA did not result in distinct clustering, a

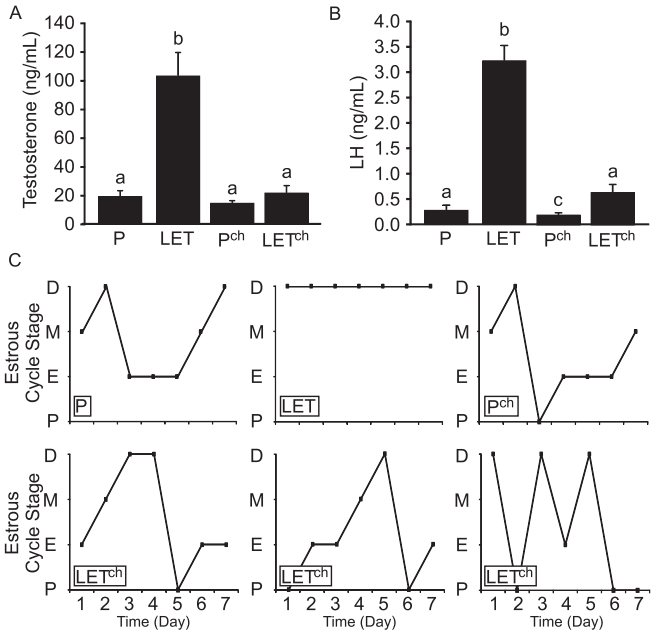

Figure 2. Letrozole mice cohoused with placebo mice did not become hyperandrogenemic or acyclic. The cohousing study included four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch}. Letrozole treatment resulted in increased (A) testosterone and (B) LH levels compared with placebo. LET^{ch} mice displayed a decrease in (A) testosterone and (B) LH and (C) a restoration of estrous cyclicity compared LET mice stuck in diestrus. Stages of the estrous cycle are indicated as diestrus (D), metestrus (M), estrus (E), and proestrus (P). Graphs illustrating the estrous cycle stages of representative mice from the four groups are shown. Graph error bars represent SEM. Different letters were used to indicate significant differences in a one-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test ($P < 0.05$).

PERMANOVA test (ADONIS) detected a significant effect of cohousing treatment on the microbial community structure ($P = 0.001$) (Fig. 6A). This trend was also observed in unweighted UniFrac (data not shown). CAP was then used to analyze the microbial composition in response to an *a priori* defined experimental variable (cohousing treatment). PERMANOVA demonstrated a strong relationship between cohousing treatment and the overall composition of the gut microbiome ($P = 0.001$) (Fig. 6B), suggesting that cohousing resulted in a distinct gut microbial community in LET^{ch} mice compared with LET mice. To understand when the gut microbiome diverged, we then compared the fecal samples from the four groups at each time point (Fig. 6C–6G). We observed a significant separation of the bacterial communities in the treatment groups after 2 weeks (ADONIS, $P = 0.004$) (Fig. 6D). Separation of the bacterial communities also occurred in weeks 3 and 4 but not in week 5, possibly because of convergence of P^{ch} and LET^{ch} with a placebo-like gut microbiome phenotype.

Differentially abundant genera are associated with cohoused letrozole mice

Differential abundance of gut bacteria between placebo- and letrozole-treated mice was determined with DESeq2. This approach used a negative binomial regression for modeling count variables and is commonly used for overdispersed data, which is typical of microbiome data (33). DESeq2 identified five bacterial genera that were of higher relative abundance and four bacterial genera that were of lower relative abundance in placebo compared with LET mice (Fig. 7A). The gram-positive bacteria included *Coprobacillus*, *Candidatus Arthromitus*, *Roseburia*, *Dorea*, *Lactobacillus*, and *Adlercreutzia*, and the gram-negative bacteria included *Akkermansia*, *Christensenella*, and *Turicibacter*. DESeq2 also identified three bacterial genera that had an altered relative abundance in LET^{ch} compared with LET mice: *Coprobacillus*, *Christensenella*, and *Lactobacillus* (Fig. 7B).

Discussion

This study demonstrated that exposure to a healthy gut microbiome resulted in protection from developing a metabolic phenotype in a PCOS mouse model. In particular, cohousing letrozole-treated mice with placebo mice resulted in LET^{ch} mice with body weight, FBG and insulin levels, and insulin resistance similar to those of placebo mice (Fig. 1). Although our results demonstrated that cohousing letrozole with placebo mice resulted in protection from metabolic dysregulation by the end of the study, future studies will be needed to ascertain how much time cohousing takes to exert a protective effect on these metabolic factors. The improved PCOS metabolic phenotype obtained via cohousing is consistent with previous cohousing studies with obese mice, in which exposure to a healthy gut microbiome provided protection from weight gain to germ-free mice inoculated with feces from obese donors (22). It is worth noting that the letrozole-induced metabolic phenotype does not involve changes in diet, food intake, or total energy expenditure (32), suggesting that other mechanisms are responsible for the development of metabolic dysregulation in PCOS. For instance, because studies have

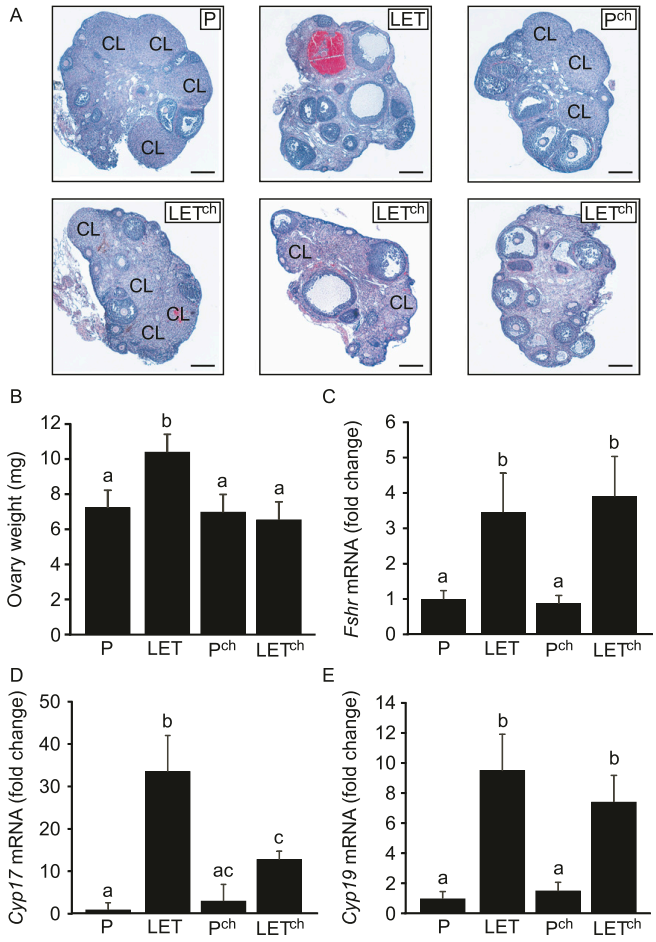

Figure 3. Cohousing letrozole mice with placebo mice improved the ovarian phenotype. The cohousing study included four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch}. (A) Letrozole treatment resulted in a lack of corpora lutea, cystlike follicles, and hemorrhagic cysts in the ovaries compared with placebo mice. (A) Unlike LET mice, LET^{ch} mice lacked polycystic ovaries, and their ovaries contained corpora lutea (CL) which is evidence of ovulation. Scale bars represent 250 μm. (B–E) Letrozole treatment also resulted in increased ovarian weight and increased mRNA expression of several ovarian genes important in ovarian follicular development and steroidogenesis. (B) Ovarian weight was lower in LET^{ch} mice compared with LET mice. *Fshr* and *Cyp19* mRNA levels were similar between LET and LET^{ch} mice, whereas *Cyp17* was lower in LET^{ch} mice compared with LET mice. Graph error bars represent standard error of the mean. Different letters were used to indicate significant differences in a one-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test ($P < 0.05$).

indicated that obesity may be influenced by an increased capacity of the gut microbiome to harvest energy from dietary fiber (21), it would be informative to test whether this occurs in letrozole-treated mice.

In addition to an effect of cohousing on the PCOS metabolic phenotype, this study reports an effect of cohousing on a reproductive phenotype. Specifically, cohousing letrozole mice with placebo mice resulted in LET^{ch} mice with normalized testosterone and LH levels as well as estrous cycling and ovarian morphology similar to those of placebo mice (Figs. 2 and 3). The presence of estrous cycles and corpora lutea in many of the LET^{ch} mice suggests that the mice were able to ovulate. Future superovulation studies could be informative in determining whether exposure to a healthy microbiome restores ovulation in letrozole-treated mice cohoused with placebo mice. Because ovarian *Cyp17* gene expression is induced by both androgens and insulin (50, 51), it is unsurprising that *Cyp17* levels were normalized in LET^{ch} mice (Fig. 3) that had reduced circulating levels of testosterone and insulin. On the other hand, it is not clear why *Fshr* mRNA levels were increased in both LET and LET^{ch} mice. With regard to aromatase (*Cyp19*) expression, one possible explanation why *Cyp19* mRNA levels did not resolve in LET^{ch} mice, despite normalization of testosterone levels, is that LET^{ch} mice were still exposed to letrozole. These results support the idea that suppression of the aromatase enzyme with letrozole treatment results in a compensatory increase in Cyp19 mRNA. This study also indicates that normalization of *Cyp19* mRNA was not necessary for an improved PCOS phenotype and suggests that the protective effect of cohousing did not occur because of decreased letrozole activity in LET^{ch} mice.

To further characterize the effects of cohousing on the letrozole-induced PCOS mouse model, we examined the effects of cohousing on the gut microbiome. As published previously (27), we observed lower α-diversity in letrozole-treated female mice compared with placebo mice. These results are consistent with studies that reported lower α-diversity of the gut microbiome in women with PCOS compared with controls (23–25). However, because we did not observe higher α-diversity in LET^{ch} mice compared with LET mice (Fig. 5), these results

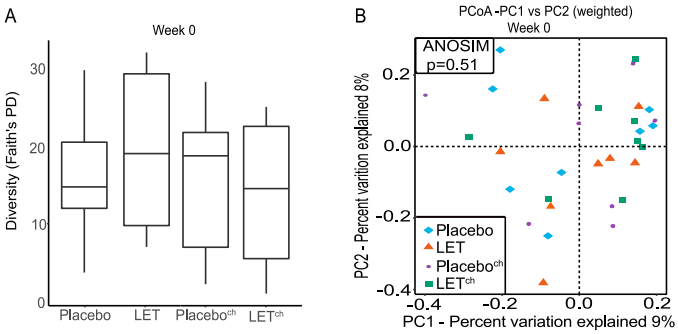

Figure 4. Gut microbiome was similar in all cohousing treatment groups before treatment. The cohousing study included four groups of mice: P, LET, P^{ch}, and LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). No significant differences in (A) gut microbial richness (α-diversity, Faith PD) or (B) community composition (β-diversity, weighted UniFrac) were observed among cohousing treatment groups before treatment (week 0; n = 8 per group). One-way ANOVA was used to compare α-diversity among the groups, and analysis of similarity (ANOSIM) test was used to compare β-diversity among the groups.

indicate that the physiological differences between LET and LET^{ch} mice are probably not due to changes in α-diversity *per se* but may reflect changes in specific gut microbes. Supporting this idea, we observed changes in the overall gut bacterial composition at the same time (Fig. 6; week 2) that we observed a protective effect of cohousing on weight gain in LET^{ch} mice (Fig. 1B). Additional support for this idea comes from the identification of specific gut bacteria such as *Coprobacillus* and *Lactobacillus* that had a relative abundance altered by letrozole treatment and restored by cohousing letrozole with placebo mice. Interestingly, these bacteria have been linked with host metabolism. *Coprobacillus* was reported to be enriched in healthy subjects compared with obese subjects and was proposed as a novel probiotic because of its association with a healthy gut microbiome (52, 53). Although *Lactobacillus* is commonly used as a probiotic, increased abundance of some *Lactobacillus* species has been reported in obese humans (54, 55), suggesting that the effect of *Lactobacillus* on metabolism may be species and strain specific (56). Our results highlight the need for bacterial species- and strain-level identification in future studies focused on the role of the gut microbiome in PCOS.

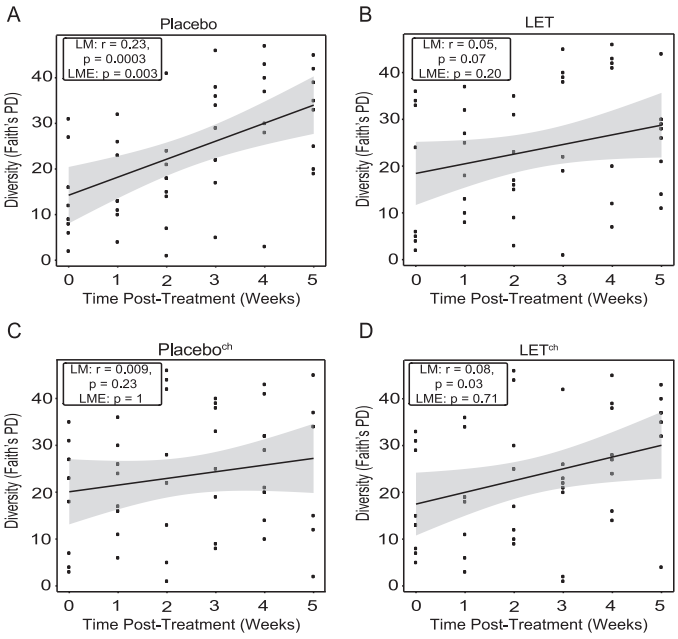

Figure 5. Cohousing letrozole mice with placebo mice did not restore α-diversity of the gut microbiome. The cohousing study included four groups of mice: (A) P, (B) LET, (C) P^{ch}, and (D) LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). α-Diversity as approximated by Faith PD ranked estimate was graphed over time for the four groups. Results of linear regression model (LM) and *P* value are in the box insets, and the gray shaded area indicates the 95% CI for the line of best fit. *P* values for the linear mixed effects model (LME) were obtained by the likelihood ratio test of the full model, with the effect in question (time) against the model without the effect in question, and are in the box insets.

Sex differences in the gut microbiome probably arise after puberty through the action of sex steroids (57). However, the mechanisms by which sex steroids influence the gut microbiome remain unclear. In the case of PCOS, previous studies in humans and mouse models of the disorder (23–25, 27) suggest that elevated testosterone levels select for different gut microbes via unknown mechanisms. Future studies investigating the role of androgen and estrogen receptors in immune cells, intestine, liver, or other relevant tissues will be important in determining whether steroid receptor signaling in the host is necessary for steroid-dependent changes in the gut microbiome. Additional studies will also be needed to determine whether androgens can directly regulate gut bacteria by acting as substrates for bacterial enzymes, such as β-glucuronidases,

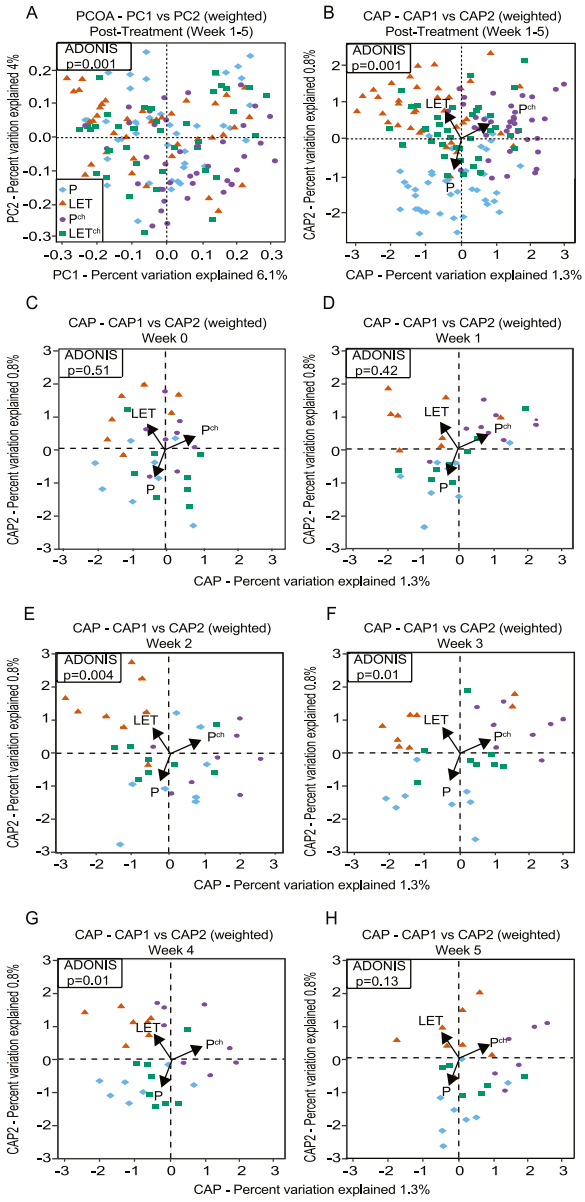

Figure 6. Cohousing letrozole mice with placebo mice influenced the overall composition of the gut bacterial community over time. The cohousing study included four groups of mice: P, LET, P^{ch}, and LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). (A) Unconstrained PCoA of weighted UniFrac distances demonstrated changes in the microbial composition (β-diversity) among samples collected after treatment. Permutational ANOVA of the weighted UniFrac distances indicated that cohousing had a strong influence on the gut microbial community ($P = 0.001$). (B) Constrained CAP of weighted UniFrac distances further illustrated the relationship between β-diversity and posttreatment with a significant effect of constraining the data based on the cohousing treatment group ($P = 0.001$). (C–H) Samples from the different groups were then compared at each time point. Permutational ANOVA of the weighted UniFrac distances was done for each time point.

important for producing carbon and energy as described for estrogens (58).

Although our results suggest that bacterial exchange may protect against the PCOS phenotype, it is possible that exposure to other fecal microbes (*e.g.*, archaea), microbial metabolites, or even steroids in feces and urine could play a protective role. Fecal microbiome transplant studies will be critical in ruling out the influence of pheromones, behavioral interactions, or urine in the protective effect of cohousing. Future reconstitution experiments will also be important to determine whether specific bacteria or metabolites are necessary for a protective effect. If bacteria prove to be an important component of the protective effect of cohousing, it is worth considering how modulating gut bacterial composition could improve reproductive function. Because studies have shown that weight loss in women with PCOS results in decreased androgen levels and improved menstrual cycling and fertility (59), it is possible that gut microbes may indirectly regulate the reproductive axis through effects on metabolism. Alternatively, gut bacteria may have a direct effect on reproduction by controlling the amount of steroid hormones excreted or reabsorbed into enterohepatic circulation through deconjugation of steroids conjugated in the liver (60). Although there are some similarities in the types of bacteria that are altered in PCOS and obesity, it remains unclear whether microbial dysbiosis and metabolic dysregulation in these two disorders result from similar mechanisms. Studies demonstrating that changes in the gut microbiome and metabolism are associated with hyperandrogenism and that PCOS metabolic dysregulation occurs in a body mass index–independent manner indicate that some of the mechanisms driving PCOS metabolic disturbances are distinct from those driving metabolic dysregulation in obesity (6, 23–25, 7).

In summary, our study demonstrated that exposure to a healthy gut

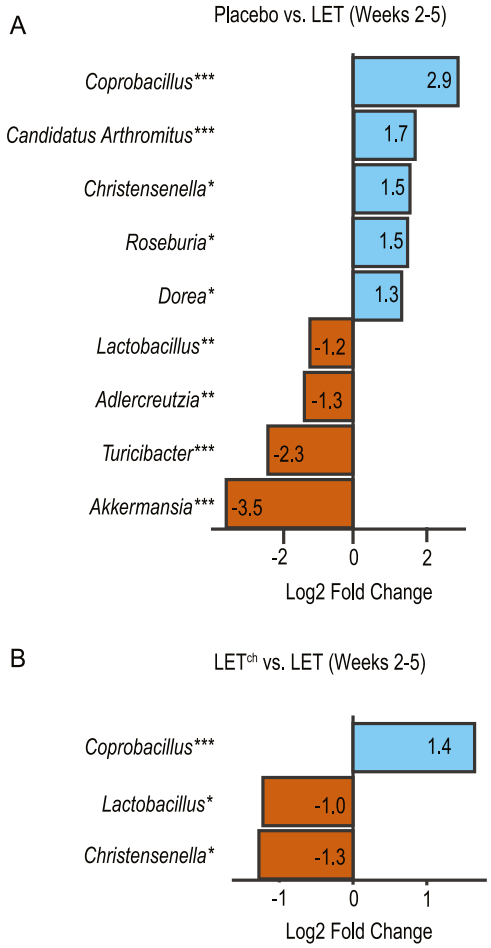

Figure 7. Specific bacterial genera were associated with improvement of the PCOS phenotype during cohousing. The cohousing study included four groups of mice: P, LET, P^{ch}, and LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). Results from the DESeq2 differential abundance analysis were expressed as log2 fold change for (A) the comparison of P and LET mice and (B) the comparison of LET^{ch} and LET mice. Positive log2 fold changes represent bacterial genera increased in (A) P mice relative to LET mice or (B) LET^{ch} relative to LET mice, whereas negative changes represent bacterial general increased in (A) LET relative to P mice or (B) LET relative to LET^{ch} mice. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

microbiome via a cohousing paradigm resulted in protection from developing metabolic and reproductive phenotypes in a letrozole-induced PCOS mouse model. The physiological phenotypes were associated with changes in the composition of the gut microbiome, suggesting that modulation of gut microbes toward a dysbiotic or healthy state may influence the degree of pathology. It is notable that cohousing also resulted in changes in the gut bacterial community of placebo mice cohoused with letrozole mice compared with placebo mice housed together. However, because these changes were not sufficient to alter the metabolic and reproductive phenotypes of the host, these results suggest that the healthy gut microbiome was resistant to any pathological influence from the feces of the letrozole mice. To elucidate how exposure to a healthy gut microbiome protected mice from developing PCOS, future studies are needed to characterize the effects of cohousing on the composition and function of the gut microbiome in the letrozole-induced PCOS mouse model by using metagenomics and metabolomics. In addition, studies are needed to investigate the mechanisms by which changes in the gut microbiome influence metabolism and reproduction. Moreover, because these results imply that modulating the composition of the gut microbiome may be a potential treatment option for women with PCOS, future studies should also investigate whether supplementation with prebiotics or novel probiotics such as *Coprobacillus* can protect against the development and pathology of PCOS.

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Chapter 3, in full, is a reprint of the material as it appears in: Torres PJ, Ho BS, Arroyo P, Sau L, Chen A, Kelley ST, Thackray VG. Exposure to a Healthy Gut Microbiome Protects Against Reproductive and Metabolic Dysregulation in a PCOS Mouse Model. *Endocrinology*. 2019;https://doi.org/10.1210/en.2019-00050. The dissertation author was the primary author of this paper.

DISSERTATION DISCUSSION AND CONCLUSION

The results from these studies have helped answer fundamental questions concerning the role of the gut microbiome in the development and pathology of PCOS. Similar to two previous studies, chapter 1 demonstrated that women with PCOS had lower microbial richness and a shift in gut microbial composition when compared to healthy women [1-4]. Through regression analysis we also showed that, similar to the letrozole-induced PCOS mouse model, hyperandrogenism was correlated with changes in the gut microbiome in women with PCOS. This is particularly impactful because these findings suggest that androgens may be an important factor in shaping the gut microbiome and that changes in the gut microbiome may influence the development and pathology of PCOS.

The clinical manifestations of PCOS often emerge around the early reproductive years suggesting that puberty is a critical period for the development and pathology of PCOS [5, 6]. Furthermore, women with PCOS who are also hyperandrogenic are likely to display a strong metabolic phenotype [7, 8]. The results in chapter 2 showed that letrozole treatment of both pubertal and adult female mice resulted in reproductive hallmarks of PCOS [9]. However, unlike pubertal mice, letrozole treatment in adult female mice resulted in only a modest PCOS metabolic phenotype and no insulin resistance. In addition, letrozole treatment of adult mice was associated with a distinct shift in the gut microbial community when compared to pubertal mice even though specific bacterial genera that changed were present in both ages. These results are important because they suggest that timing of excess androgen exposure may be an important component in the development of the PCOS metabolic phenotype and associated changes in the gut microbiome.

In recent years, several human and rodent studies have reported that the gut microbiome of individuals with PCOS differs significantly from healthy individuals and suggest that microbial imbalance or "dysbiosis" in the gut may contribute to the pathology of PCOS [1-4, 10-12]. Using a PCOS mouse model, we addressed whether the gut microbiome contributes to the pathophysiology of PCOS and if manipulation of the gut microbiome can be used to treat PCOS. The results in chapter 3 significantly added to the microbiome and PCOS field by being the first to report that the reproductive and metabolic phenotype of PCOS improved with exposure to heathy gut bacteria [13]. These results suggest that altering the gut microbiome via pre-/probiotic therapies or introduction of bioactive molecules may be a potential treatment option for women with PCOS.

While the chapters in this thesis focused on the bacterial part of the gut microbiome, it is possible that other members of the microbiome (archaea, virus, fungi) also play a significant role in the pathophysiology of PCOS. Throughout the chapters, we emphasized the need to move beyond the genera level typically obtained through 16S rRNA gene sequencing and into the strain level using shotgun metagenomics. This approach will also yield fruitful information regarding other members of the gut microbiome and the genes they carry with them.

Nevertheless, if bacteria are involved in the pathophysiology of PCOS, what are the mechanisms involved in this process? In order to better understand their potential mechanisms, we must first understand current hypotheses pertaining to PCOS pathology. PCOS is one of the most poorly understood medical disorders, possible reasons for this include the perceived inadequacy of its naming, its heterogenous nature and the uncertainty about its etiology and pathophysiology [14, 15]. What is known is that obesity is a common

feature of PCOS and up to 80% of women with PCOS are reported to be overweight or obese in the U.S. [16]. Obesity can exacerbate many of the reproductive and metabolic abnormalities associated with PCOS. Obesity is associated with increased insulin resistance and hyperinsulinemia which are also common findings in PCOS [16, 17]. Insulin resistance and hyperinsulinemia contribute to androgen excess in PCOS by stimulating ovarian androgen production, decreasing serum sex hormone-binding globulin and modulating luteinizing hormone pulsatility [18, 19]. One hypothesis suggests that abdominal adiposity together with insulin resistance contribute to ovarian and adrenal hyperandrogenism in women with PCOS [20, 21]. However, insulin resistance can occur independent of obesity. Furthermore, PCOS is not universal in women presenting with insulin resistance and insulin resistance is not universal in PCOS either [15]. Another hypothesis on the pathophysiological mechanisms underlying PCOS, and one that most closely resembles what we see in the letrozole-induced PCOS mouse model, suggest that a primary resulting in androgen excess is essential for the development of this syndrome [22]. This theory suggests that a vicious cycle of androgen excess favoring abdominal and visceral adiposity facilitates insulin resistance, which in turn further facilitates androgen secretion by the ovaries and adrenal glands in women with PCOS [15, 20, 23, 24].

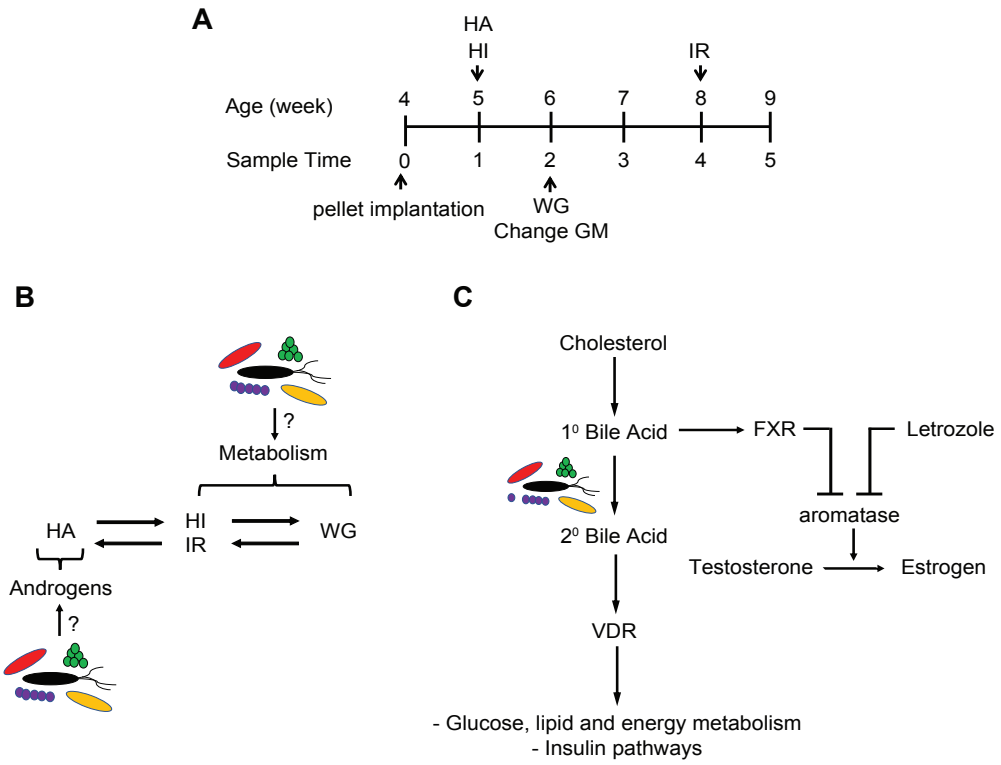

Figure 4-1: Timeline of the emergence of metabolic (hyperinsulinemia – HI; weight gain – WG; insulin resistance – IR) and gut microbiome changes (gut microbiome – GM) in a hyperandrogenic (HA) letrozole induced polycystic ovary syndrome (PCOS) mouse model (A). Hypothesis explaining the emergence of the PCOS phenotype as the result of a vicious cycle in which androgen excess favors hyperinsulinemia facilitating weight gain, the weight gain in combination with hyperinsulinemia gives rise to insulin resistance and perpetuation of hyperandrogenism (B). Two possible mechanisms that the gut microbiome could regulate the PCOS phenotype is via modulation of androgen levels or modulation of host metabolism (B). Hypothesis explaining how bile acids produced by the host (1^0) and modified by the gut microbiota (2^0) can act as ligands for the farnesoid X receptor (FXR) and vitamin D receptor (VDR) and affect the PCOS metabolic phenotype (C).

Previous work in our lab has demonstrated that hyperandrogenism (HA) and hyperinsulinemia (HI) occurs after 1 week of letrozole treatment, weight gain (WG) and changes in the gut microbiome (GM) occur by week 2 and insulin resistance (IR) occurs by week 4 [10, 13, 25, 26] (Figure 4-1A). I hypothesize that a vicious cycle of androgen excess (HA) favoring hyperinsulinemia (HI) facilitates weight gain (WG), the weight gain in combination with the hyperinsulinemia eventually gives rise to insulin resistance (IR) perpetuating hyperandrogenism in our PCOS mouse model (Figure 4-1B). In this cycle, there

are two possible ways that the gut microbiome could have an effect on the PCOS phenotype: 1. direct modulation of androgen levels or 2. modulation of the host metabolism (Figure 4-1B).

In light of these considerations, I hypothesize that the mechanism by which the gut microbiome improves the PCOS phenotype is via modulation of the host metabolism. In fact, the first-line treatment option for overweight/obese women with PCOS is diet and lifestyle interventions [27, 28]. Weight loss in PCOS women has been shown to ameliorate the clinical signs and symptoms of PCOS including hyperandrogenism and insulin resistance [29], menstrual dysfunction [30] and oligoovulation [31]. Strategies for managing PCOS symptoms also include targeting insulin resistance through the use of an insulin-sensitizer such as metformin. Metformin has similar effects to lifestyle interventions in terms of weight loss but is superior in its effects on decreasing androgen concentrations [32]. Studies have shown that changes in the gut microbiome are associated with metabolic disorders such as obesity, type 2 diabetes and insulin resistance [32-34]. Moreover, studies have reported that transplantation of stool from obese donors into germ free mice results in an obese phenotype, suggesting that the gut microbiome may play a causative role in metabolic dysregulation. Interestingly, a recent study showed that metformin's ability to increase insulin sensitivity was through the gut microbiome and its metabolic metabolites [34].

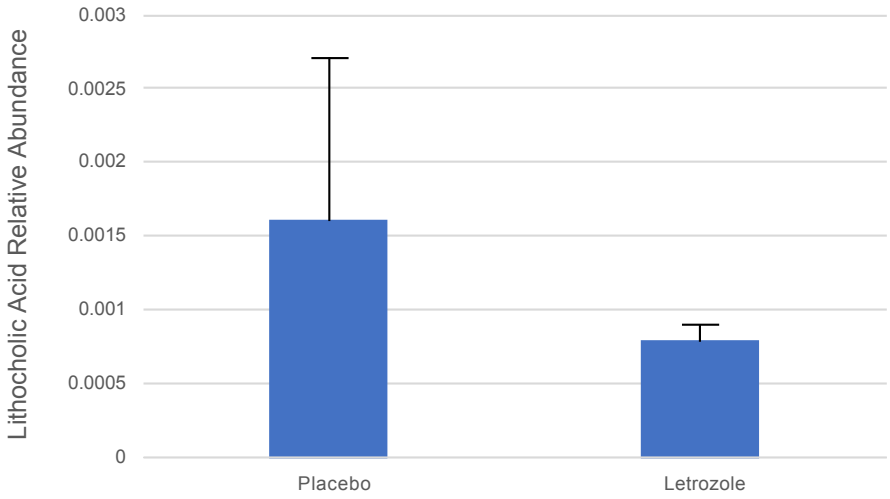

Figure 4-2: Relative abundance of lithocholic acid in the feces of placebo and letrozole treated mice at the end of 5 weeks of treatment.

The gut microbiome not only facilitates harvesting of nutrients and energy from the diet but also produces numerous metabolites capable of activating host receptors which can regulate several host processes, including metabolic processes. One such class of metabolites are produced in the liver from cholesterol initially forming primary bile acids which can then be metabolized by gut microbiome specific enzymes (e.g. bile salt hydrolases – BSH) forming secondary bile acids. Bile acids facilitate fat digestion and absorption and play an integral role in shaping the gut microbiota and host physiology [35]. Based on our preliminary work using untargeted metabolomics, we noted that a microbially derived secondary bile acid (lithocholic acid) differed significantly between placebo and letrozole mice after 5 weeks of treatment (Figure 4-2). Since bile acids have been associated with obesity [36] and insulin resistance [37], it is interesting to consider the role that they might play in PCOS.

Secondary bile acids, such as lithocholic acid, can activate vitamin D receptor (VDR) mediating vitamin D signaling [38]. Vitamin D plays important roles in metabolic pathways affected by PCOS, including insulin resistance and dyslipidemia [39-41]. Vitamin D

concentration has been found to be negatively correlated with the severity of the disorder [42]. Studies investigating the effect of vitamin D supplementation on women with PCOS have reported improvement of the metabolic and reproductive phenotype; however, these results have been mixed and they require further randomized interventional studies on a larger group of patients [43-47].

Nevertheless, an altered bile acid pool composition has been implicated in the pathogenesis of several metabolic diseases [35, 36, 48, 49]. With regards to PCOS, primary bile acids have been shown to be positively associated with hyperandrogenism in women with PCOS [50]. Primary bile acids can function as signaling molecules by binding to the farnesoid X receptor (FXR) [51-53]. FXR plays key roles in bile acid homeostasis and acts as a metabolic regulator [54]. Interestingly, primary bile acids have been shown to down regulate the conversion of androgens to estrogens by activating FXR, which further inhibited the expression and activity of aromatase [55]. This suggest that increased primary bile acids might act on the aromatase enzyme already being interfered with by letrozole there by exacerbating the hyperandrogenism in the letrozole-induced PCOS mouse model. In essence, gut microbes could reduce inhibition of the aromatase by shifting the bile acid pool away from primary into secondary bile acids capable of influencing host metabolism leading to an improved PCOS phenotype (Figure 4-1C).

Given the association between excess primary bile acids and hyperandrogenism in PCOS, future studies should attempt to orally administer gut microbiota-derived bile salt hydrolase (BSH) into our mouse model. This could help rebalance the bile acid pool by metabolizing the primary bile acids and forming secondary bile acids. If imbalances in the primary and secondary bile acid pool are contributing to the PCOS phenotype, I would expect

to see amelioration of the PCOS phenotype in letrozole-treated mice given BSH. Primary bile acids are known to directly interact with the FXR receptor; however, it is unclear if the FXR is playing a role in PCOS. Future studies could administer letrozole to mice with an intestine-specific knockout of FXR to determine whether activity of this receptor in the intestine is required for the PCOS metabolic phenotype and changes in the gut microbiome.

In addition to exploring the role of bile acids and FXR in the pathology of PCOS, it is also important to determine whether the gut microbiome is necessary and sufficient for the development of PCOS. Future experiments using a letrozole-induced PCOS germ free mouse model or the introduction of letrozole to pubertal mice given broad-spectrum antibiotics will be critical. If the gut microbiome is sufficient for the development of the PCOS phenotype, then we would not expect mice with depleted gut microbiomes to develop the PCOS phenotypes. However, recapitulation of the PCOS phenotype in germ free mice or mice treated with broad-spectrum antibiotics would indicate that the gut microbiome is not necessary for PCOS development but could be sufficient to protect from the PCOS phenotype. Fecal microbiome transplant of a healthy mouse gut microbiome into the letrozole-treated germ free or broad-spectrum antibiotic treated PCOS mouse model and treatment with pre/probiotics or metabolites will be critical in determining if modulation of the gut microbiome is a viable treatment option for women with PCOS.

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