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RESEARCH ARTICLE

Temporal relationship between alterations in the gut microbiome and the development of polycystic ovary syndrome-like phenotypes in prenatally androgenized female mice

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Abstract

It has been recently recognized that prenatal androgen exposure is involved in the development of polycystic ovary syndrome (PCOS) in adulthood. In addition, the gut microbiome in adult patients and rodents with PCOS differs from that of healthy individuals. Moreover, recent studies have suggested that the gut microbiome may play a causative role in the pathogenesis of PCOS. We wondered whether prenatal androgen exposure induces gut microbial dysbiosis early in life and is associated with the development of PCOS in later life. To test this hypothesis, we studied the development of PCOS-like phenotypes in prenatally androgenized (PNA) female mice and compared the gut microbiome of PNA and control offspring from 4 to 16 weeks of age. PNA offspring showed a reproductive phenotype from 6 weeks and a metabolic phenotype from 12 weeks of age. The α-diversity of the gut microbiome of the PNA group was higher at 8 weeks and lower at 12 and 16 weeks of age, and the β-diversity differed from control at 8 weeks. However, a significant difference in the composition of gut microbiome between the PNA and control groups was already apparent at 4 weeks. *Allobaculum* and *Roseburia* were less abundant in PNA offspring, and may therefore be targets for future interventional studies. In conclusion, abnormalities in the gut microbiome appear as early as or even before PCOS-like phenotypes develop in PNA mice. Thus, the gut microbiome in early life is a potential target for the prevention of PCOS in later life.

Abbreviations: AGD, anogenital distance; ASV, amplicon sequence variant; DHT, dihydrotestosterone; ELISA, enzyme-linked immunosorbent assay; FBG, fasting blood glucose level; ITT, insulin tolerance testing; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; NGS, next-generation sequencing; OTU, operational taxonomic unit; PC, principal coordinate; PCoA, principal coordinate analysis; PCOS, polycystic ovary syndrome; PCR, polymerase chain reaction; PNA, prenatally androgenized.

KEYWORDS
androgens, delayed effects of prenatal exposure, gastrointestinal microbiome, polycystic ovary syndrome

1 | INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of reproductive-age women, affecting 6%–20% of this group.[1] PCOS is diagnosed using the Rotterdam criteria, which require two of the following to be present: hyperandrogenism, oligo-anovulation, and polycystic ovarian morphology. However, PCOS is a heterogeneous and complex disorder that has adverse reproductive and metabolic implications for the affected women; the pathology involves abnormal gonadotropin secretion, altered ovarian morphology and function, and disordered insulin action.[2]

It has recently been shown that the gut microbiome of individuals with metabolic disorders, such as obesity and type 2 diabetes, differs from that of healthy individuals and that this may play a causative role in the development of metabolic disorders.[3,4] When considered together with the findings that sex steroid hormone concentrations are related to gut microbial composition,[5,6] it is plausible that changes in the gut microbiome may play a role in the pathology of PCOS.[7] Indeed, it was recently reported that the gut microbiome of adult patients with PCOS differs from that of healthy individuals.[8–10] In addition, rodent models of PCOS that are generated by the long-term administration of androgens or aromatase inhibitors to pubertal or adult animals have a different gut microbiome in adult animals when compared with controls.[11–14] Moreover, the transplantation of feces from or co-housing with healthy controls ameliorates the PCOS-like phenotype of rodent models. Thus, the gut microbiome may play a role in the pathogenesis of PCOS.[15,16]

It has been recently recognized that prenatal androgen exposure is involved in the development of PCOS in adulthood. Women with PCOS are hyperandrogenic during pregnancy and their daughters are at a high risk of subsequently developing PCOS.[17–19] In addition, prenatally androgenized (PNA) animals, including rodents, sheep, and rhesus monkeys, exhibit PCOS-like endocrine and metabolic phenotypes in adulthood.[19,20]

On the basis on these findings, we hypothesized that prenatal exposure to high concentrations of androgens would induce dysbiosis of the gut microbiome early in life and that this would lead to the development of PCOS in later life. As a first step in testing this hypothesis, in this study, we aimed to characterize the development of PCOS-like phenotypes in PNA female mice and to compare the gut microbiomes of PNA and control female offspring. It is known that adult PNA offspring exhibit PCOS-like reproductive and metabolic phenotypes,[19,21–24] but the temporal changes in the phenotype of the PNA offspring remain to be fully characterized. To this end, we analyzed the gut microbiomes of PNA and control mice when they were prepubertal, at puberty, and during their adolescence, young adulthood, and adulthood, then identified the temporal relationship between alterations in the gut microbiome and the development of PCOS-like phenotypes. Our results show that abnormalities appear in the gut microbiome as early as or even before PCOS-like phenotypes manifest in PNA offspring.

2 | MATERIALS AND METHODS

2.1 | PNA model

The PNA model was generated by the injection of pregnant mice with dihydrotestosterone (DHT), as previously reported.[19,21–24] Eight-week-old male and female C57BL/6 mice, aged 8–12 weeks, were provided by Japan SLC Inc (Hamamatsu, Japan). The mice were maintained under specific pathogen-free conditions and a 12-h light/12-h dark cycle, with ad libitum provision of water and feed. After 1 week's adaptation to their surroundings, the female mice were paired with male mice and subsequently checked for the presence of copulatory plugs. The date of identification of a plug was recognized as day 1 of gestation. The experimental design is shown in Figure 1. Pregnant dams were subcutaneously injected on days 16, 17, and 18 of gestation with either 0.1 ml sesame oil (control group) or 0.1 ml sesame oil containing 250 μg of DHT (Sigma-Aldrich, St. Louis, MO, USA; PNA group).[19,21,22,24] The female pups of the control and PNA dams ($n = 60$, respectively) were weaned 4 weeks postnatally, and housed separately from their dams after weaning. The female offspring of the control and PNA dams were housed separately, with three or four pups per cage. The PCOS phenotype and gut microbiome of the female offspring in both groups were then analyzed at 4, 6, 8, 12, and 16 weeks of age ($n = 12$ per time point); the offspring of the control and PNA dams were randomly allocated to analyze at five time points, respectively. These ages correspond to prepuberty, puberty, adolescence, young

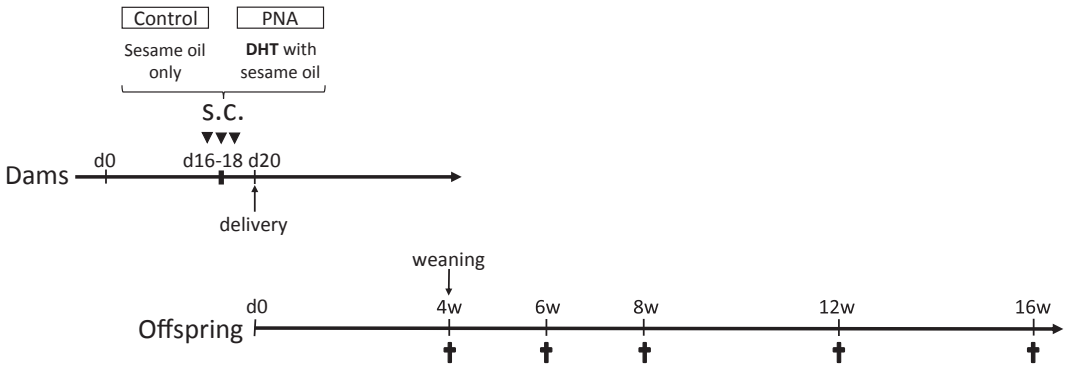

FIGURE 1 Experimental design. The scheme shows how prenatally androgenized (PNA) offspring were generated and how the outcomes were evaluated. Pregnant dams were subcutaneously (s.c.) injected on days 16, 17, and 18 of gestation with either 0.1 ml sesame oil (control group) or 0.1 ml sesame oil containing 250 μg of dihydrotestosterone (DHT) (PNA group). The female pups in the control and PNA groups ($n = 60$ each) were weaned at 4 weeks of age. The evaluation of the PCOS phenotype and gut microbiome of the female offspring was performed at 4, 6, 8, 12, and 16 weeks of age ($n = 12$ each). The development of a PCOS-like reproductive phenotype was identified by considering estrous cyclicity, ovarian histology, and serum testosterone concentration; and the development of the metabolic phenotype was identified by considering body weight, the size of visceral adipocytes, the results of insulin tolerance testing (ITT), and fasting blood glucose levels (FBG)

adulthood, and adulthood, respectively.[25] Anogenital distance (AGD), a distance from the center of the anus to the posterior fourchette, was measured early in their lives, as an indicator of fetal androgen exposure. PNA offspring had longer AGD than control offspring: 4.59 ± 0.11 vs. 3.46 ± 0.10 mm ($p < .001$) at 4 weeks and 5.53 ± 0.08 vs. 3.94 ± 0.09 mm ($p < .0001$) at 6 weeks, confirming that PNA offspring was exposed to androgen in utero. Mice were euthanized under isoflurane anesthesia, and blood, ovarian, parametrial fat, and fecal samples were collected. The PCOS-like reproductive phenotype was identified by estrous cyclicity, ovarian histology, and serum testosterone concentration; and the metabolic phenotype was determined by body weight, the size of visceral adipocytes, insulin tolerance testing (ITT), and fasting blood glucose levels (FBG). All the procedures used in the study were performed in compliance with the guidelines and regulations of the University of Tokyo Committee on the Use and Care of Animals, and the study was approved by the committee (approval number: P21-005).

2.2 | Onset of puberty and estrous cyclicity

The effects of PNA on the onset of puberty and estrous cyclicity were determined using well-established methods.[22,26] The onset of puberty was identified by determining the age at which the vaginal introitus opened and estrous cyclicity began. To evaluate estrous cyclicity, vaginal cells were collected from female offspring every morning, beginning 1 week before the mice were sacrificed. The stage of the estrous cycle was categorized as diestrus, proestrus, estrus, or metestrus using light microscopy, as previously described.[27,28] Briefly, each stage of the estrous cycle was defined according to the presence and proportions of neutrophils, nucleated epithelial cells, and anuclear keratinized epithelial cells. In diestrus, neutrophils predominate; in metestrus, neutrophils and anuclear keratinized epithelial cells are present; in estrus, anuclear keratinized epithelial cells predominate; and in proestrus, small, round, nucleated epithelial cells are visible.

2.3 | Ovarian and adipocyte histology

To assess the effect of PNA on ovarian and visceral adipocyte histology, ovaries and parametrial fat were collected from control and PNA mice at 4, 6, 8, 12, and 16 weeks of age. The ovaries and adipose tissue were fixed, embedded in paraffin, and 5-μm-thick sections were prepared. These sections were stained with hematoxylin and eosin. Ovarian sections were analyzed by two investigators using an Olympus BX50 Fluorescence Microscope (Olympus, Tokyo, Japan). The number of atretic antral follicles and corpora lutea were counted in every sixth section across the entire ovary, as previously described.[29] Atretic follicles were recognized by the presence of an attenuated granulosa cell layer, shrinkage, and degenerate oocyte nuclei.[30] For adipose tissue, two representative micrographs were taken per sample at a 40× magnification using a light microscope (Olympus BX50 Fluorescence Microscope; Olympus). The size of adipocytes was quantified by using ImageJ software as previously described.[31]

2.4 | Serum testosterone concentration

To determine the serum testosterone concentration, blood samples were collected from control and PNA mice at 4, 6, 8, 12, and 16 weeks of age by cardiac exsanguination under isoflurane anesthesia at 09:00, because testosterone concentrations exhibit diurnal variations. The samples were centrifuged and the separated serum was stored at −80°C until assayed. The serum concentration of testosterone was measured using an ELISA kit (RRID: AB_2848196; ENZO Life Sciences, Farmingdale, NY, USA).

2.5 | Insulin tolerance testing (ITT) and fasting blood glucose levels (FBG)

To evaluate insulin resistance, ITT was performed as previous described.[32] Briefly, mice were administered an intraperitoneal injection of insulin 0.75 IU/kg body weight after fasting for 5 h, then the blood glucose concentrations of blood samples from a tail vein were measured using a glucometer (LAB Gluco, ForaCare Japan, Tokyo, Japan) 0, 15, 30, 60, 90, and 120 min later. FBG, another marker of insulin resistance, was measured after overnight fasting.

2.6 | DNA extraction and next-generation sequencing (NGS) of fecal bacterial 16S rRNA genes

Fecal samples were collected from the control and PNA groups at 4, 6, 8, 12, and 16 weeks of age, and stored at −80°C. A DNA extraction kit (NucleoSpin DNA stool, Takara Bio Inc, Kusatsu, Japan) was used to extract DNA from these samples. Two-step PCRs were performed on the purified DNA to prepare sequencing libraries using a 16S (V3–V4) Metagenomic Library Construction Kit for NGS (Takara). The first PCR amplification was performed using the primer pair 341 F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 806 R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3′) with Illumina adaptor overhang sequences. The second PCR amplification was performed using the Nextera XT Index kit v2 (Illumina, San Diego, CA, USA). Sequencing libraries were purified using the Agencourt AMPure XP (Beckman Coulter) and quantified by fluorescence using the Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Clonal clusters of the libraries were generated and sequenced on a MiSeq system (Illumina) with the MiSeq Reagent v3 kit in 2 × 250 bp mode.

2.7 | Bioinformatic analysis of fecal bacterial 16S rRNA genes

16S rRNA analysis was performed using QIIME2 (version: 2019.7).[33,34] First, the demultiplexed sequencing data was denoised using the DADA2 plugin. Amplicon sequence variants (ASVs) obtained from DADA2 were clustered into operational taxonomic units (OTUs) at 99% identity using the vsearch plugin. Next, taxonomy was assigned to OTUs against the Greengenes 99% OTU database (version 13.8) using the scikit-learn plugin. The relative abundances at the taxonomic level from kingdom to species were determined based on OTU tables. OTUs were also classified against DDBJ database for 16S ribosomal RNA. After taxonomic classification, OTUs were aligned and masked using the MAFFT plugin with the default parameter. A rooted phylogenetic tree was built with FastTree plugin. Alpha-diversity and beta-diversity analyses were performed using the q2-diversity plugin. Beta-diversity analysis was performed using weighted and unweighted UniFrac distances calculated among samples at the sampling depth of 10,000 reads. Moreover, principal coordinate analysis (PCoA) was performed to visualize similarities of each sample. Linear discriminant analysis (LDA) effect size (LEfSe) was performed to identify the bacterial taxa with significant difference between control and PNA groups. The data have been deposited with links to BioProject accession number PRJDB12175 in the DDBJ BioProject database.

2.8 | Statistical analysis

Student's *t*-test was used to compare the PCOS phenotypes of the control and PNA groups at the same time points, with JMP Pro 15 software (SAS Institute Inc, Cary, NC, USA). The statistical analyses for alpha- and beta-diversity and microbial composition were performed using the QIIME (version: 1.8.0). The comparison in alpha-diversity was performed using nonparametric *t*-test with Monte Carlo permutations with Benjamini–Hochberg FDR correction through the "compare_alpha_diversity.py" QIIME script with parameter setting of -t nonparametric -n 999 -p fdr -d 50 000. The tests of significance in beta-diversity were performed using nonparametric *t*-test with Monte Carlo permutations with Bonferroni correction through the "make_distance_boxplots.py" QIIME script with parameter setting of -n 1000 -t two-sided. In LEfSe analysis, a significant value of <.05 and LDA effect size of >2 were used as thresholds. Relationships between continuous variables were assessed using Pearson's correlation coefficient. The data are shown as means ± SEMs and $p < .05$ was accepted as indicating statistical significance.

3 | RESULTS

3.1 | PNA offspring show a delay in puberty and disruption to their estrous cycle at all of the ages assessed

All the control offspring showed vaginal patency and regular estrous cycling at the age of 6 weeks. By contrast, a significantly smaller number of PNA offspring (6/12, $p = .0016$ vs. control) showed vaginal patency at the same age, which suggests that the onset of puberty is delayed in PNA offspring. Moreover, the number of vaginal cells collected from PNA offspring at 6 weeks of age was very small, even in those with vaginas that had opened. Therefore, the evaluation of the estrous cycle became possible in the PNA group from 7 weeks of age. PNA offspring exhibited disruption to their estrous cycles at all the time points (Figure 2).

3.2 | PNA offspring show histology typical of polycystic ovaries and increased serum testosterone concentrations from 6 weeks of age

Figure 3A shows representative histological images of ovaries from mice of both groups. Follicle counting revealed a significant higher number of atretic antral follicles in the PNA group than in the control group at the ages of 6, 12, and 16 weeks (Figure 3B). The numbers of corpora lutea were significantly decreased in PNA offspring at 8, 12, and 16 weeks (Figure 3C).

To determine whether PNA affected serum testosterone concentration, this was measured at each time point and compared between the control and PNA groups. As shown in Figure 4, there was no significant difference between the groups at 4 weeks of age; however, the PNA offspring had significantly higher testosterone concentrations than the controls at 6, 8, and 12 weeks, and showed a similar trend at 16 weeks ($p = .060$).

3.3 | PNA offspring show low body weight until adolescence, but high body weight, hypertrophy of visceral adipocytes, and insulin resistance from 12 weeks of age

PNA offspring had lower body weight than controls at the ages of 4 and 8 weeks and showed the same trend at 6 weeks of age ($p = .080$). By contrast, 12-week-old PNA offspring were significantly heavier than controls, and there was a similar trend at 16 weeks of age ($p = .080$). These results suggest that PNA reduces birth weight but that the weight gain of the offspring increases after adolescence, resulting in higher body weight in the young adult and adult mice (Figure 5A). In parallel, parametrial adipocytes were significantly larger

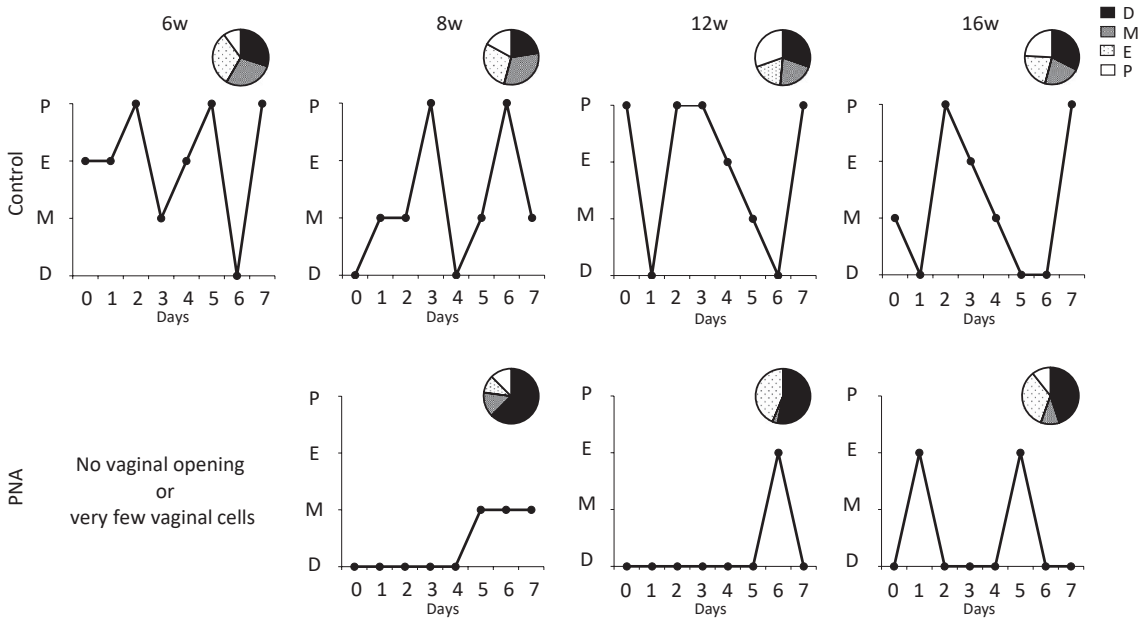

FIGURE 2 Prenatally androgenized (PNA) offspring show disruptions in estrous cycling at all the assessed ages. Representative estrous cycles for control (upper panels) and PNA (lower panels) mice are shown. PNA offspring showed no vaginal patency or very few vaginal cells to evaluate their estrous cycling if their vaginas were patent at 6 weeks of age. Therefore, the assessment of the estrous cycle became possible from 7 weeks. The pie graphs show the data of % in each stage of estrous cycle of all mice in corresponding groups. PNA offspring exhibited disruption of their estrous cycles at all the time points. D, diestrus; M, metestrus; E, estrus; P, proestrus

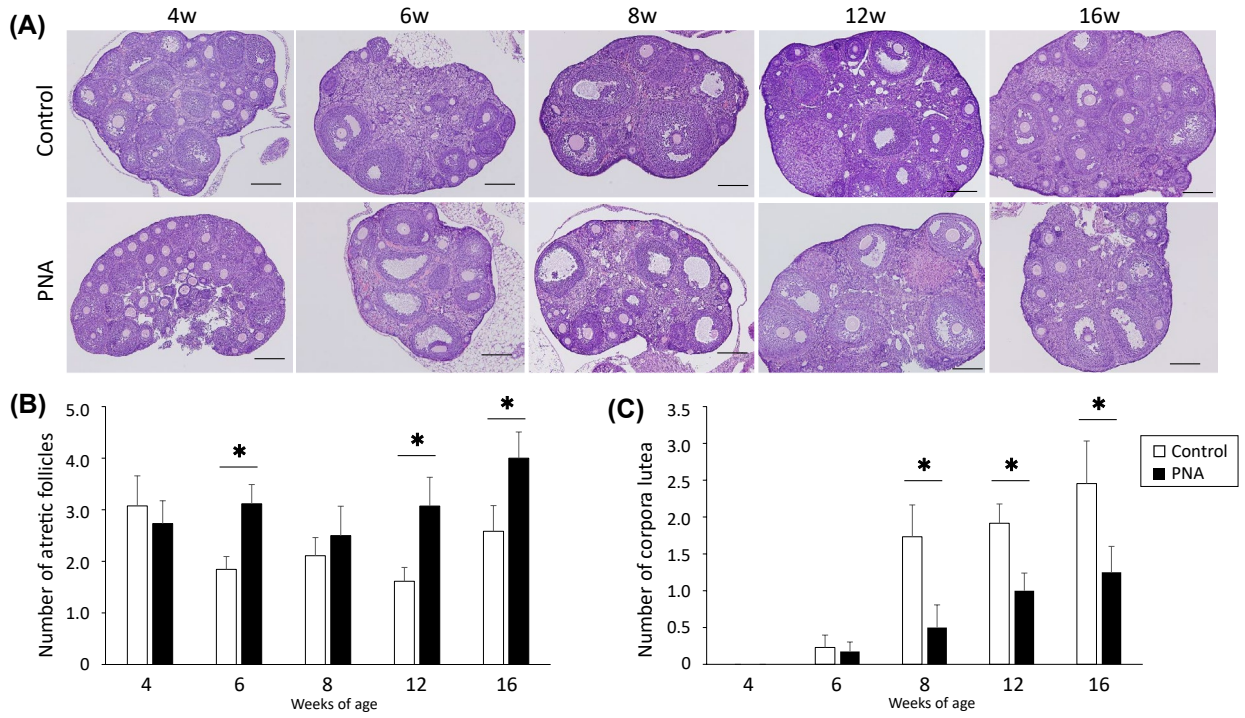

FIGURE 3 Prenatally androgenized (PNA) offspring show histology typical of polycystic ovaries from 6 weeks of age. (A) Representative sections of an ovary, (B) the number of atretic antral follicles, and (C) the number of corpora lutea in the control and PNA groups at 4, 6, 8, 12, and 16 weeks of age. From 6 weeks (puberty), cystic follicles with large antra and degenerate granulosa cells are apparent in ovaries from PNA offspring. The numbers of corpora lutea were significantly decreased in PNA offspring after 8 weeks, suggesting ovulation was impaired. Scale bars: 400 μm. Values are means ± SEMs. *$p < .05$ vs. the control group at each time point

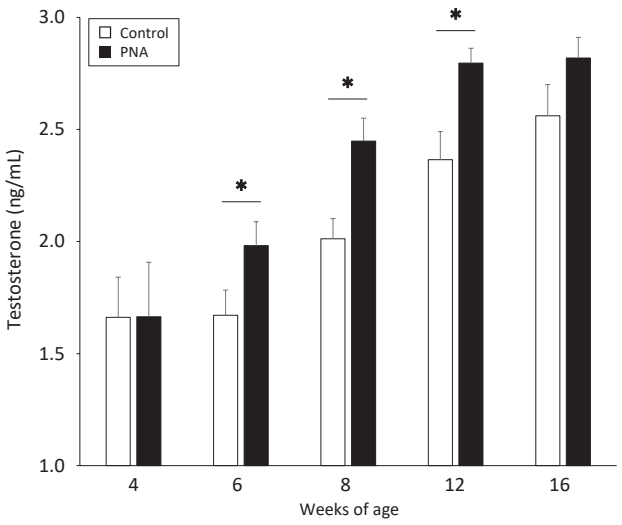

FIGURE 4 Prenatally androgenized (PNA) offspring show increased serum testosterone concentration from 6 weeks of age. The serum testosterone concentrations of control and PNA offspring were measured at 4, 6, 8, 12, and 16 weeks. The concentration gradually increased with age in both groups, but was significantly higher in PNA offspring than in control offspring from 6 weeks of age. Values are means ± SEMs. *$p < .05$ vs. the control group at each time point

in PNA offspring at 12 and 16 weeks than controls, while significantly smaller at 6 weeks (Figure 5B,C).

Figure 6A shows the results of ITT. The insulin-induced suppression of serum glucose was weaker between 90 and 120 min after the insulin injection in 12-week-old PNA offspring, and in 16-week-old offspring it was weaker at 60 and 90 min. Blood glucose area under the curve (AUC) during ITT was significantly increased in PNA offspring at 12 and 16 weeks, but did not differ between PNA and control offspring at 4, 6, and 8 weeks (Figure 6B). Furthermore, PNA offspring showed significantly elevated FBG at 6, 12, and 16 weeks compared with control offspring (Figure 6C). These findings imply lower insulin sensitivity in PNA offspring at 12 and 16 weeks of age.

3.4 | The alpha-diversity of the gut microbiome is significantly affected by PNA at 8, 12, and 16 weeks of age

The α-diversity, which reflects the richness of the microbial species, was assessed using the number of OTUs and the Chao1 index. The number of OTUs in the PNA group was significantly higher than that of the control group at

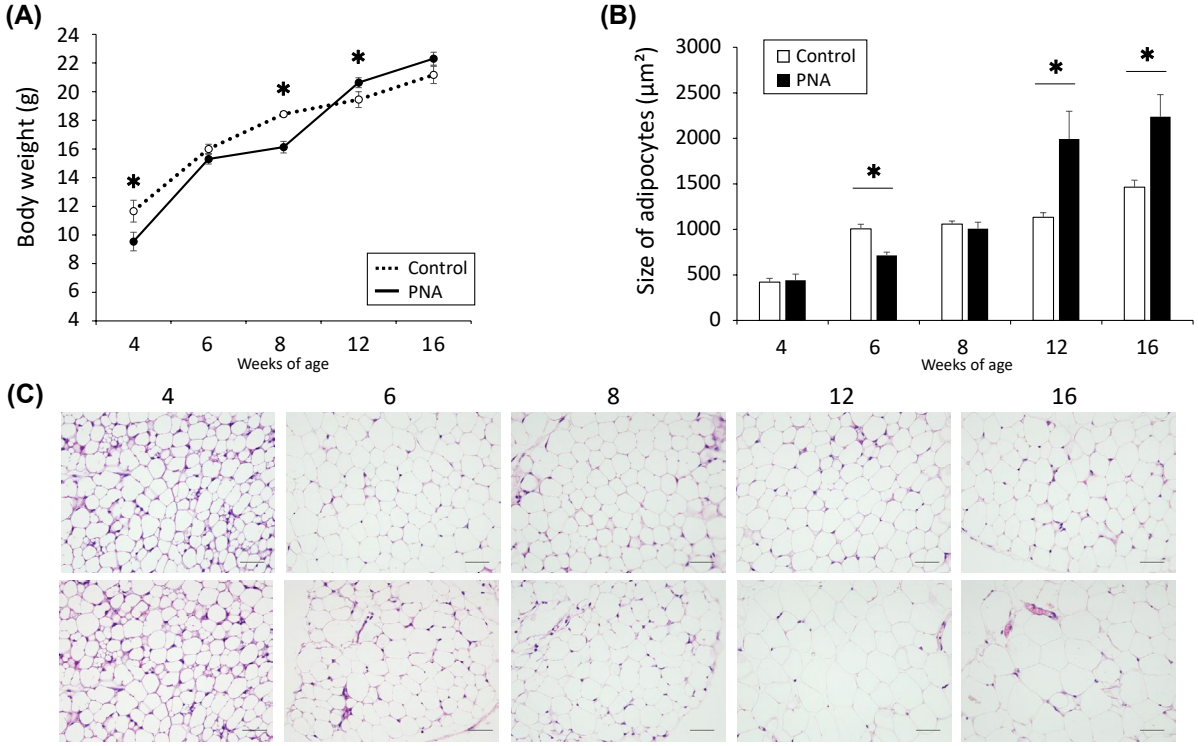

FIGURE 5 Prenatally androgenized (PNA) offspring are heavier and show hypertrophy of parametrial adipocytes from 12 weeks of age. (A) The body weights of control and PNA offspring were measured at the ages of 4, 6, 8, 12, and 16 weeks. PNA offspring showed lower body weight until 8 weeks, but weight gain increased after 8 weeks, such that they showed higher body weight from 12 weeks (young adulthood) onwards. (B) The size of parametrial adipocytes was measured in control and PNA offspring. In parallel with the change in body weight, PNA offspring showed hypertrophy of parametrial adipocytes at 12 and 16 weeks. (C) Representative sections of parametrial adipocytes, 40× magnification. Scale bars: 50 μm. Values are means ± SEMs. *$p < .05$ vs. the control group at each time point

8 weeks, but lower at 12 and 16 weeks of age. Similarly, the Chao1 index of the PNA group was significantly higher at 8 weeks, but significantly lower at 12 weeks, and tended to be lower at 16 weeks of age ($p = .055$) (Figure 7).

3.5 | The beta-diversity of the gut microbiome is significantly affected by PNA at 8 weeks of age

The β-diversity, which reflects the similarity between groups, was assessed using the weighted UniFrac distance and visualized using PCoA. The PCoA plots showed a significant separation of the PNA group from the control group at 8 weeks of age (Figure 8), which implies that the microbial communities of the two groups substantially differed at this time point.

3.6 | The composition of the gut microbiome differs between PNA and control offspring at all the ages assessed

The gut microbial composition of control and PNA offspring was analyzed taxonomically at the levels of bacterial phyla and genera. The identification of the bacterial taxa with statistically significant difference between control and PNA offspring was performed using LEfSe software and LDA. At the phylum level, the gut microbiome was mainly composed of Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria at each of the time points (Figure 9), which is consistent with previous human and rodent data.[7,35] As shown in Figure 10, the Firmicutes were significantly more abundant in the PNA group at 4 and 8 weeks of age, but significantly less abundant at 16 weeks. The Proteobacteria was less abundant in the PNA group at 4 weeks of age. Finally, the Actinobacteria were significantly less abundant at 6 and 8 weeks of age.

The abundances of several bacterial genera significantly differed between the control and PNA groups at each time point (Figure 10). Table 1 is a summary of the overall differences in microbial composition between the two groups, and lists all the bacterial taxa that were consistently more or less abundant in the PNA group across multiple time points. Most of them were less abundant in the PNA group: *Gemella*, *Allobaculum, Clostridium, Roseburia, Coprococcus,* f__[Mogibacteriaceae];g__, f__Ruminococcaceae;g__ of the phylum Firmicutes; *Sutterella* of the phylum Proteobacteria; and *Adlercreutzia* of the

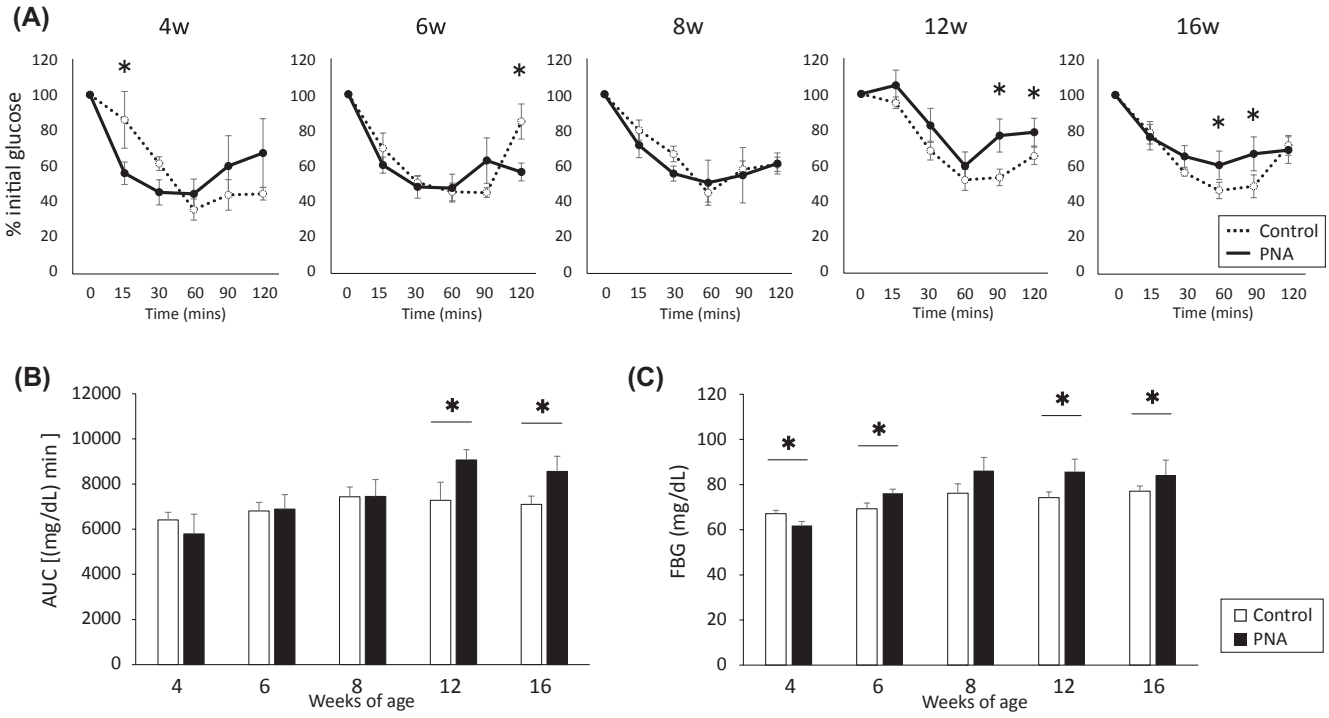

FIGURE 6 Prenatally androgenized (PNA) offspring are insulin resistant from 12 weeks of age. (A) Insulin tolerance testing (ITT) was performed at 4, 6, 8, 12, and 16 weeks. The insulin-induced suppression of serum glucose was weaker between 90 and 120 min after the insulin injection in 12-week-old PNA offspring, and in 16-week-old offspring, it was weaker at 60 and 90 min. (B) Area under the curve (AUC) during ITT. AUC was significantly increased in PNA offspring compared to control offspring at 12 and 16 weeks. (C) Fasting blood glucose levels (FBG) were measured after overnight fasting. FBG were significantly increased in PNA offspring compared to control offspring at 6, 12, and 16 weeks. These findings imply lower insulin sensitivity in PNA offspring at 12 and 16 weeks of age. Values are means ± SEMs. *$p < .05$ vs. the control group at each time point

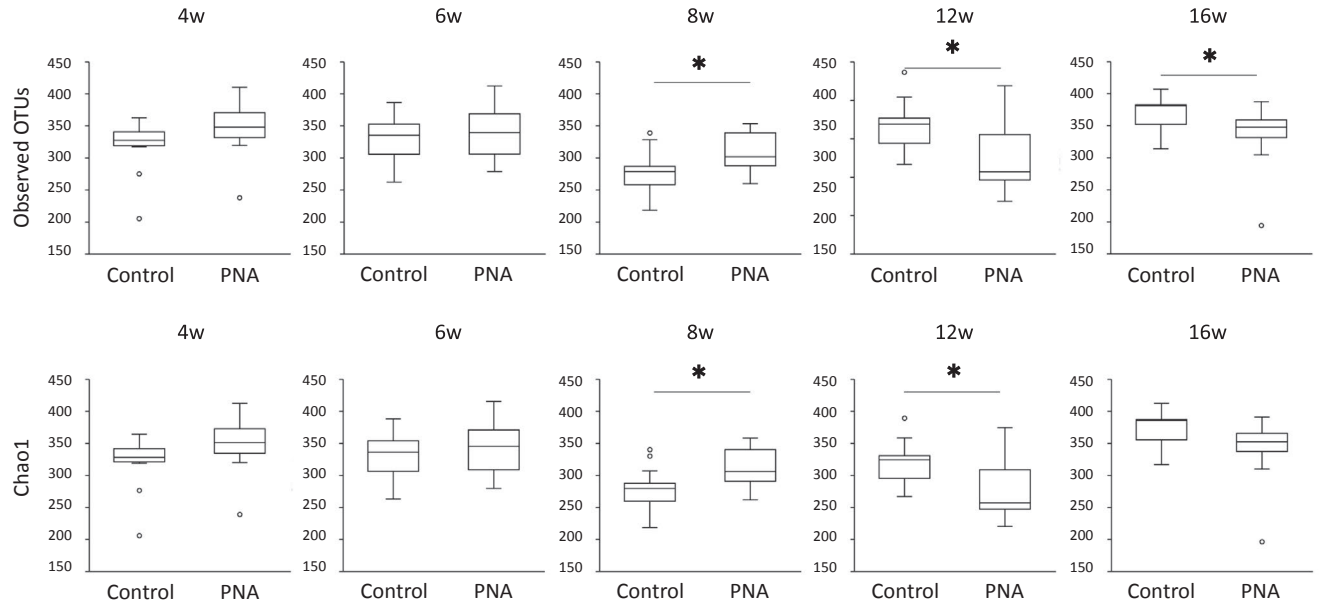

FIGURE 7 The α-diversity of the gut microbiome is significantly different in prenatally androgenized (PNA) and control offspring at 8, 12, and 16 weeks of age. Two indicators of α-diversity, the number of operational taxonomic units (OTUs) and the Chao1 index, are shown for the PNA and control groups in the upper and lower panels, respectively. The comparison in α-diversity was performed using nonparametric *t*-test with Monte Carlo permutations with Benjamini–Hochberg FDR correction. The α-diversity was higher in the PNA group at 8 weeks and lower at 12 and 16 weeks. *$p < .05$ vs. the control group at each time point

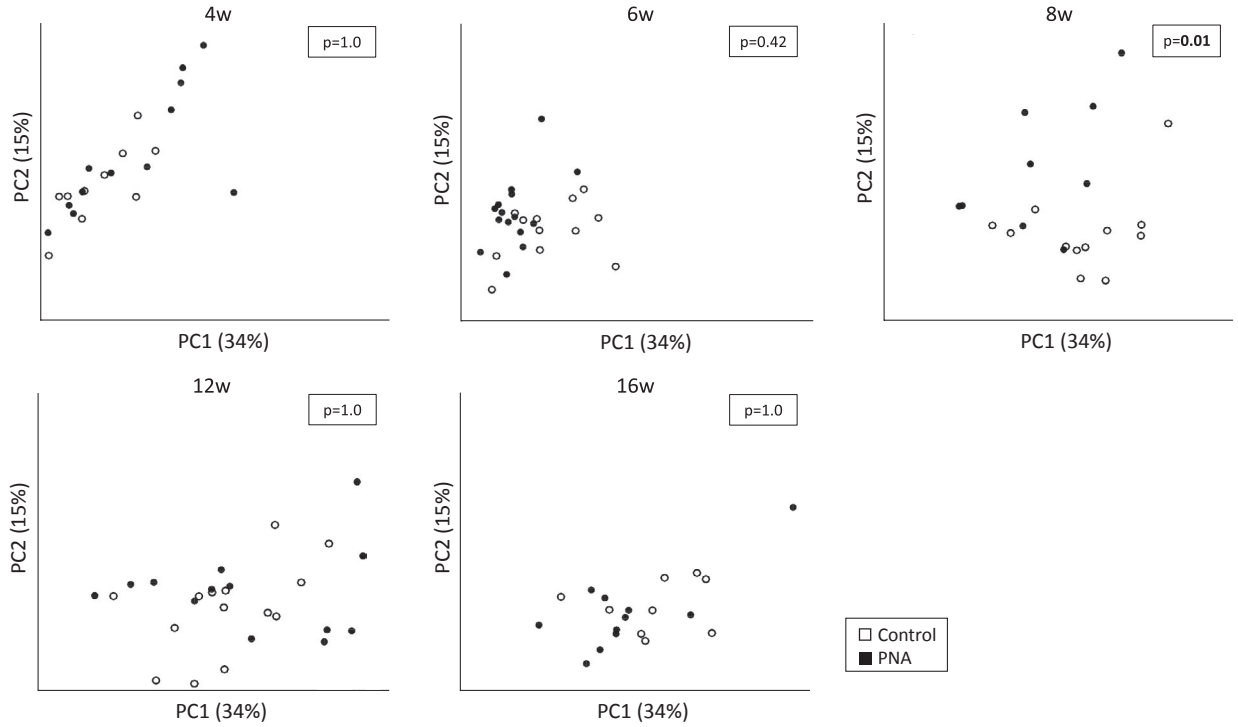

FIGURE 8 The β-diversity of the gut microbiome is significantly different in prenatally androgenized (PNA) and control offspring at 8 weeks of age. The β-diversity, reflecting the similarity between the groups, was analyzed at each time point using a principal coordinate analysis (PCoA) plot that was based on the weighted UniFrac metric. Each dot represents the composition of the bacterial community in an individual sample. The proportions of the variance that could be explained using principal coordinate (PC) axes 1 and 2 were 34% and 15%, respectively. The tests of significance in β-diversity were performed using nonparametric *t*-test with Monte Carlo permutations with Bonferroni correction. The microbial community of the PNA offspring could be discriminated from that of the control offspring at 8 weeks of age

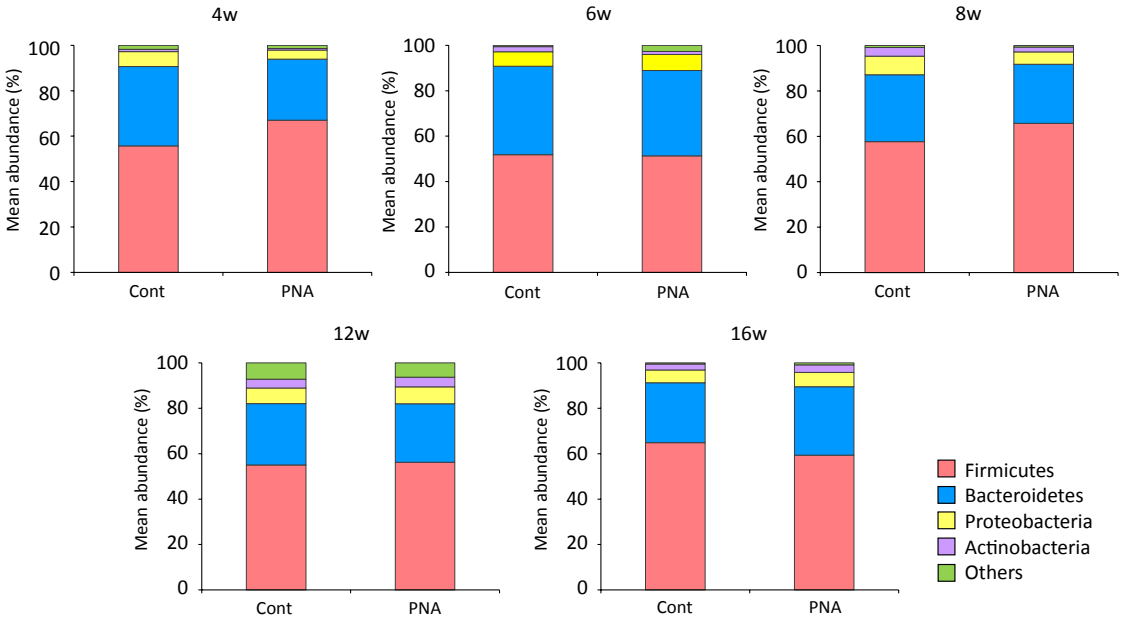

FIGURE 9 Overall structure of the gut microbiome at the phylum level. The microbial population structure at the phylum level is shown. The microbiome was principally composed of four phyla: the Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, at each time point

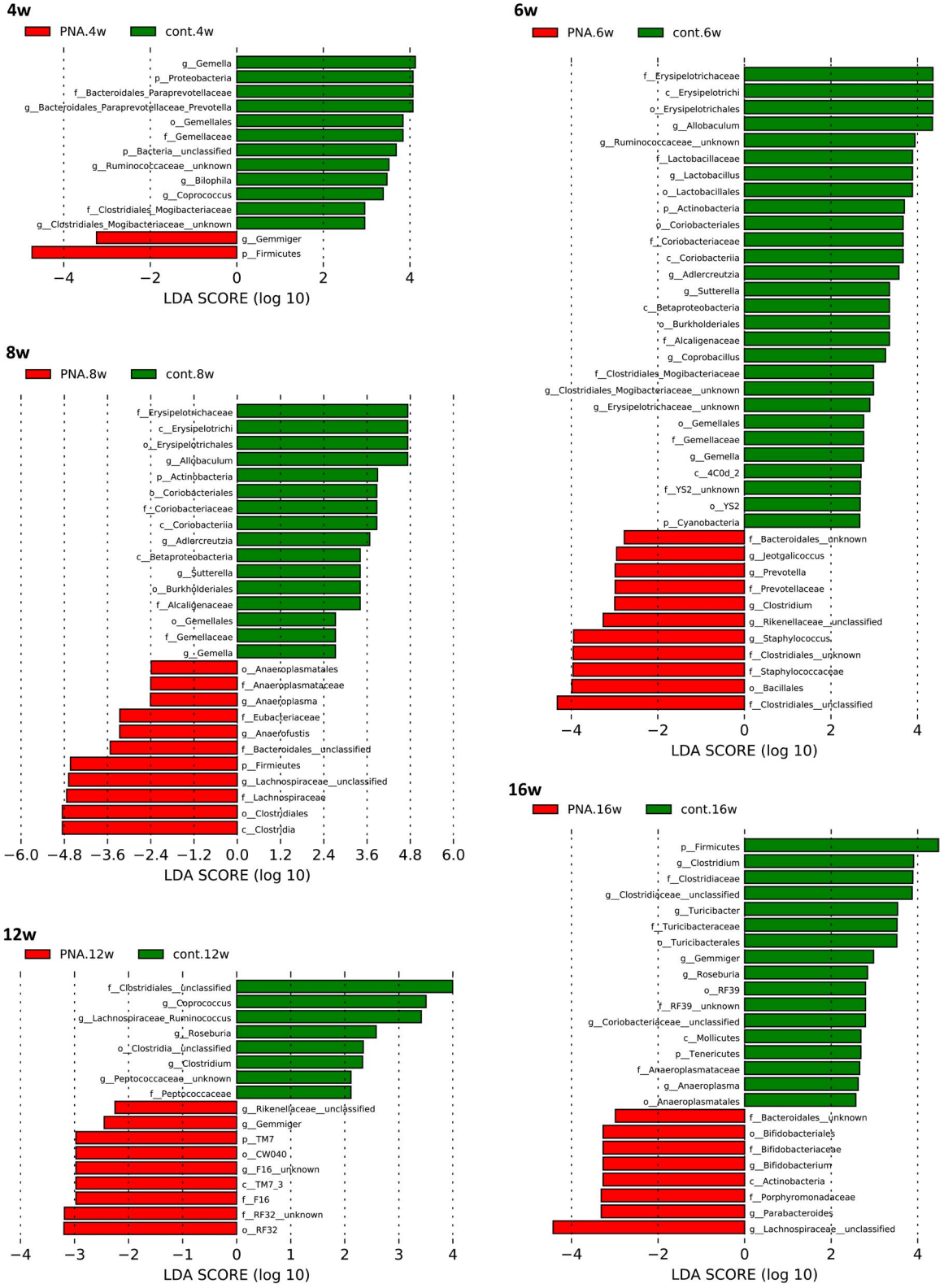

FIGURE 10 The composition of the gut microbiome differed in prenatally androgenized (PNA) and control (cont) offspring at every time point. Differentially abundant taxa between control and PNA offspring were identified using linear discriminant analysis (LDA) effect size (LEfSe) software. Taxa enriched in control and PNA groups are colored by green and red, respectively. A significant value of <.05 and LDA effect size of >2 were used as thresholds in LEfSe analysis. Differences in microbial composition were identified in multiple taxa at each time point. Characteristic species in control and PNA offspring were already apparent from 4 weeks of age (prepuberty) and persisted until 16 weeks of age (adulthood)

TABLE 1 Differences in the gut microbial composition of the prenatally androgenized (PNA) and control groups

Phylum	Genus	4 weeks	6 weeks	8 weeks	12 weeks	16 weeks
Firmicutes	f__[Mogibacteriaceae];g__	⇩	⇩			
	Gemella	⇩	⇩	⇩		
	f__Ruminococcaceae;g__	⇩	⇩			
	Gemmiger	⇧			⇧	⇩
	Allobaculum		⇩	⇩		
	Clostridium		⇧		⇩	⇩
	Roseburia				⇩	⇩
	Coprococcus	⇩			⇩	
Bacteroidetes	f__Rikenellaceae;__		⇧		⇧	
Proteobacteria	*Sutterella*		⇩	⇩		
Actinobacteria	*Adlercreutzia*		⇩	⇩		

Notes: All the bacterial taxa that were consistently more or less abundant in the PNA group across multiple time points, identified by linear discriminant analysis (LDA) effect size (LEfSe), are summarized. In LEfSe analysis, a log LDA score >2.0 and $p < .05$ were set as the thresholds. Upward and downward arrows indicate higher and lower abundance in the PNA group than in the control group, respectively.

phylum Actinobacteria. On the other hand, *Gemmiger* of the phylum Firmicutes and f__Rikenellaceae;__ of the phylum Bacteroides were more abundant in the PNA group. Of interest, these changes were already observed early in life: at 4 weeks in *Gemella*, *Coprococcus,* f__[Mogibacteriaceae];g__, f__Ruminococcaceae;g__, and *Gemmiger*; at 6 weeks in *Gemella*, *Allobaculum, Sutterella, Adlercreutzia,* f__[Mogibacteriaceae];g__, f__Ruminococcaceae;g__, *and* f__Rikenellaceae;__; and at 8 weeks in *Gemella*, *Allobaculum, Sutterella,* and *Adlercreutzia*. Among them, *Allobaculum* was of interest because its relative abundance was considerably high: 4% at 6 weeks and 20% at 8 weeks in the control group. *Roseburia* was another taxon of interest, because it was absent in all the PNA offspring throughout the study, whereas the number of mice carrying the genus increased in the control group according to the age: one of 12 at 4, 6, and 8 weeks; five of 12 at 12 weeks; and six of 12 at 16 weeks of age.

4 | DISCUSSION

We have shown that PNA delays puberty in female mouse offspring and subsequently disrupts their estrous cycle. Polycystic ovarian histology and increased serum testosterone concentration were identified from puberty onwards. In addition, PNA reduced the body weight of the offspring until adolescence, implying fetal growth restriction. However, from young adulthood onwards, the female pups showed higher body weight, hypertrophy of visceral adipocytes, and insulin resistance. Furthermore, PNA affected the α-and β-diversity of the gut microbiome: the former was higher during adolescence, but lower from young adulthood onwards, and the latter differed in PNA and control offspring during adolescence. Altered composition of the gut microbiome was apparent before puberty and was present at all the time points thereafter. In particular, *Allobaculum* was less abundant at puberty and during adolescence in the PNA offspring; and *Roseburia* was absent in PNA offspring. Figure 11 summarizes the findings of this study.

First, we assessed the reproductive and metabolic features of PCOS in PNA offspring between 4 weeks (prepuberty) and 16 weeks (adulthood). The finding that their estrous cycle was disrupted from 7 weeks of age, when we were first able to assess this, until adulthood is consistent with those of previous studies.[22–24,36,37] Similarly, histological findings compatible with PCOS, such as a large number of atretic antral follicles, are common findings in adult PNA offspring.[19,23,36,37] Specifically, we found larger numbers of atretic follicles at puberty (6 weeks), and during adolescence (8 weeks), young adulthood (12 weeks), and adulthood (16 weeks), although statistical significance was not achieved at 8 weeks of age. The findings are consistent with those of previous studies, although very few have characterized the ovarian histology of the offspring before they reached adulthood.[22,38,39] Decreased numbers in corpora lutea in PNA offspring from 8 weeks onwards suggested that polycystic ovarian morphology was accompanied by impaired ovulation. Increased serum testosterone concentration was identified in PNA offspring from puberty (6 weeks) until adulthood. In most previous studies, increased testosterone concentration was identified in adulthood,[22,26,40] and the youngest age at which increased testosterone concentration had been identified in PNA offspring was 8–9 weeks.[39] The increase in testosterone levels in PNA offspring was modest, that is, less than twofold,

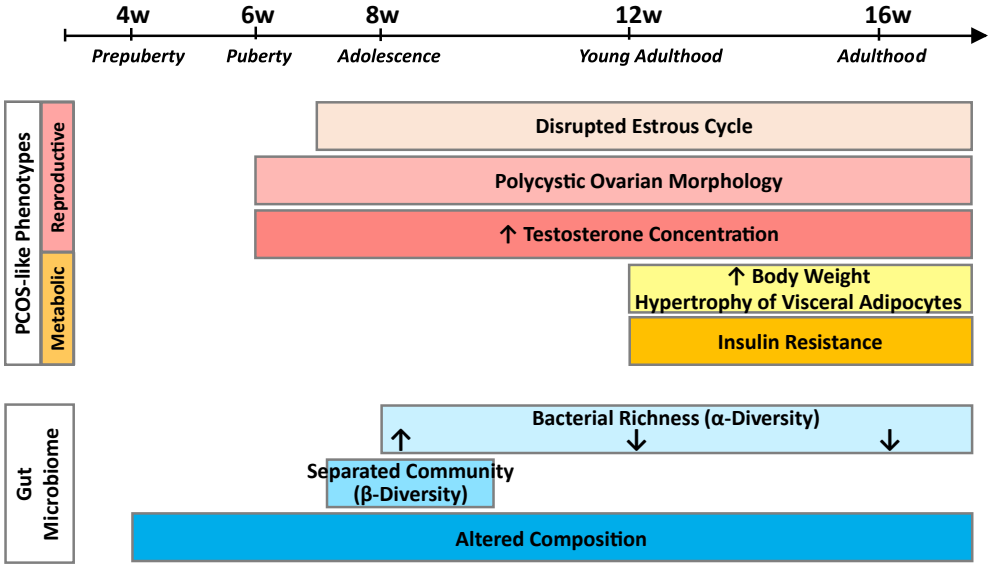

FIGURE 11 Development of polycystic ovary syndrome (PCOS)-like phenotypes and changes in the gut microbiome in PNA offspring during their growth and development. A reproductive phenotype including disruption of the estrous cycle, histology typical of PCOS, and increased serum testosterone concentration were apparent at puberty in PNA female offspring. A metabolic phenotype, including obesity, hypertrophy of visceral adipocytes, and impaired insulin sensitivity, became apparent in young adulthood. With respect to the gut microbiome, the α-diversity, reflecting species richness, in the PNA female offspring was higher at 8 weeks, and lower at 12 and 16 weeks of age, than in the control offspring. The microbial community of the PNA offspring, evaluated using β-diversity, discriminated it from that of control offspring at 8 weeks of age. A significant difference between the PNA and control groups in the composition of gut microbiome was already apparent at 4 weeks and remained thereafter

compared to controls both in this study and previous studies; modest increase in testosterone levels seems to be characteristic of PNA offspring from puberty onwards. Puberty was delayed in the PNA offspring in this study, but previous studies have generated inconsistent findings in this regard: some have shown delayed onset and others early onset.[22,26,37,40] Alterations in the secretion of luteinizing hormone and neuropeptides in the hypothalamus of PNA offspring may affect the timing of puberty.[36]

In comparison to the reproductive phenotype, the metabolic phenotype has been poorly explored. In this study, we have shown that PNA results in lower birth weight but greater weight gain after adolescence, such that the offspring have higher body weight in young adulthood (12 weeks) and adulthood (16 weeks). Simultaneous with the appearance of higher body weight, hypertrophy of visceral adipocytes and lower insulin sensitivity were also identified in the offspring. There have been few reports of the body weight of young female PNA offspring and the results have conflicted.[22,26,37,38] The body weight of the adult PNA offspring did not differ from that of the control group in previous studies.[23,36,40,41] However, in one study, the adipocyte area in parametrial fat and the degree of steatosis were higher in adult PNA female offspring than in controls, despite a lack of difference in body weight.[23] Finally, the glucose metabolism of PNA female offspring has rarely been studied, using FBG, fasting insulin levels, glucose tolerance testing, or ITT, either in adulthood or in younger offspring, and the results have been inconsistent.[19,23,24,38,40]

In summary, as shown in Figure 11, the reproductive phenotype of PCOS, aspects of which are used as diagnostic criteria, including hyperandrogenism, irregular menstrual cycle (disrupted estrous cycle in rodents), and polycystic ovarian morphology, were faithfully recapitulated in this study and became apparent at puberty in the PNA offspring, although the increase in testosterone levels was modest compared to that usually observed in PCOS patients. By contrast, the metabolic characteristics, such as obesity, and impaired insulin sensitivity, became apparent in young adulthood. The finding of this study that the increase in testosterone levels precedes weight gain and insulin resistance is consistent with the finding made using another PCOS mouse model, which is generated by long-term treatment with letrozole, an aromatase inhibitor, that hyperandrogenemia is present between 4 and 9 weeks of age.[42]

The α-diversity of the PNA group was higher at 8 weeks and lower at 12 and 16 weeks. Low α-diversity indicates low species richness. Multiple studies of the human gut microbiome have demonstrated lower α-diversity in premenopausal women with PCOS than in healthy women.[8,9,43,44] In addition, a recent study demonstrated that adolescent girls (14–16 years old) with PCOS and

obesity had lower α-diversity than girls with obesity but no PCOS.[45] Two previous studies have characterized the gut microbiome of adult PNA female offspring: one showed that the α-diversity of the gut microbiome of 22-week-old mice was lower than that of controls,[46] whereas the other showed that α-diversity was higher in 25-week-old mice.[47] Interestingly, in mice in which PCOS was induced by long-term postnatal treatment with letrozole, the effects on the gut microbiome were dependent on the age at which the letrozole was administered. If the treatment was performed between 4 and 9 weeks of age, the α-diversity was subsequently lower, whereas if it was performed between 8 and 13 weeks of age, the α-diversity was unaffected.[11,12] The finding of this study that α-diversity was low after young adulthood, at 12 and 16 weeks, is consistent with those of previous human studies and studies of rodents exposed to letrozole between 4 and 9 weeks of age. It is conceivable that low α-diversity after young adulthood has a role in the generation of the metabolic phenotype, including obesity and insulin resistance, which was identified during the same period, given that a low α-diversity has been associated with manifestations of metabolic abnormalities, such as obesity and type 2 diabetes.[48,49] Although higher α-diversity has been identified in males than females after puberty,[10] higher α-diversity in PNA offspring at 8 weeks in this study could not be solely attributable to the increased testosterone concentration that is present from 6 weeks onwards, given that the testosterone levels in PNA offspring were only 1.2-fold higher than those in control offspring. Future studies will elucidate another factor that may affect α-diversity in PNA offspring at 8 weeks.

The β-diversity differed between PNA and control groups at 8 weeks of age. Differences in the microbial community of adult patients with PCOS and healthy control, indicated by differences in β-diversity, have been identified in humans.[8,9,43,44] Adolescent girls (14–16 years old) with PCOS and obesity also showed differing β-diversity to girls with obesity but no PCOS.[45] In adult PNA rodents, the results of similar studies have been conflicting—there was no difference in PNA mice, whereas there was a difference in PNA rats.[46,47] Mice with PCOS generated by long-term postnatal treatment with letrozole showed altered β-diversity, independent of the age at which the treatment started.[11,12] In this study, the difference between the microbial community in the PNA and control groups was marked at 8 weeks. The microbial community of PNA offspring at 8 weeks of age was similar to those of PNA and control offspring at 4 and 6 weeks, whereas the microbial community of the control offspring at 8 weeks was more similar to those of PNA and control offspring at 12 and 16 weeks. Therefore, we speculate that the acquisition of an adult-like microbial community may be delayed in PNA offspring.

Altered composition of the gut microbiome, at both the phylum and genus levels, of PNA female offspring was apparent as early as 4 weeks and continued throughout the study. Various taxa have been reported to be up- or downregulated in adult rodents with PCOS, depending on the model used, and in adult women with PCOS.[43] To the best of our knowledge, this study is the first to determine the gut microbiome of such a model early in their lives. Notably, the findings of this study are in accordance with recent findings made when adolescent girls with PCOS and obesity were compared with adolescent girls with PCOS but without obesity.[45] Thus, although the gut microbiome of humans and rodents differ, alterations in their compositions appear relatively early in the life of both rodent models and women who develop PCOS.

Of the taxa that were found to be different in the gut microbiome of PNA offspring compared to control offspring, the *Allobaculum* and *Roseburia* of the phylum Firmicutes may represent potential targets of future interventional studies. We have shown that the relative abundance of *Allobaculum* is significantly lower in 6- and 8-week-old PNA offspring than in control offspring. This lower abundance of *Allobaculum* may have considerable effects on the overall gut microbial composition, because its relative abundance was high in the control group. Interestingly, there was a positive correlation between the abundances of *Allobaculum* and *Sutterella* of the phylum Proteobacteria which was also significantly less abundant in 6 and 8-week-old PNA offspring (Table 1). There have been no previous studies in which *Allobaculum* itself was administered; however, several types of treatment, such as insulin-sensitizing drugs, plant-derived supplements, and probiotics, which have beneficial effects in rodent models of various types of pathology, including obesity, dyslipidemia, and colitis, increase the relative abundance of *Allobaculum* in the gut microbiome.[50–52] In addition, it was recently reported that dietary α-linolenic acid-rich flaxseed oil ameliorates the PCOS-like phenotype in a rat model generated by treatment with letrozole during adolescence, and this was accompanied by an increase in the relative abundance of *Allobaculum*.[53] In this study, we have shown that *Roseburia* is absent in PNA offspring throughout the period of assessment, unlike in the control offspring, which generally acquired bacteria of this genus. Accumulating evidence indicates that *Roseburia* is associated with host health in humans. For example, decrease in relative abundance of *Roseburia* has been identified in various types of disease, including obesity, type 2 diabetes, irritable bowel syndrome, and neurodegenerative diseases.[54–56] In addition, *Roseburia* is less abundant in adult patients with PCOS, as well as in adolescent patients with PCOS and obesity.[9,45,57,58] Thus, *Roseburia* is expected to be one of the next-generation probiotics,

although its use to date has been limited to models of animal disease.[55]

There are some limitations in this study. One is the difference of the gut microbiome between humans and rodents, in spite of high similarities at the phylum level between the murine and human gut microbiomes.[35] Accordingly, findings in rodents, especially at the genus level, cannot be directly applied to humans, although similar changes were observed in the gut microbiome of both adult PCOS patients and model mice, such as lower α-diversity than controls.[43] In addition, the gut microbiome of rodent models varies according to the model applied to the experiments, and furthermore, none of the currently available models completely recapitulates human PCOS pathology. In spite of these inherent limitations of the studies using model rodents, it is plausible that the findings of this study, that is, alterations in the gut microbial composition of individuals who develop PCOS appear relatively early in life, are applicable to the pathophysiology of PCOS in humans, when considered together with the findings in adolescent girls with PCOS.[45] Another limitation is that the mechanism which causes the difference between the gut microbiome of the offspring of control and PNA dams was not addressed in this study. Altered intrauterine environment of PNA dams induced by DHT injection might affect the gut microbial formation of their pups. Alternatively, the gut/skin/milk microbiome of PNA dams, altered by DHT treatment, might be transmitted to their pups during delivery and breastfeeding. Future studies comparing the gut microbiome of the dams and their pups would be helpful to address this point.

In conclusion, prenatal exposure to high androgen concentrations, which mimic the in utero environment of women with PCOS, affects the gut microbial composition of female offspring, not only in adulthood, but also during the prepubertal and pubertal periods and adolescence. In addition, the reproductive characteristics of PCOS become apparent at puberty and the metabolic characteristics appear in young adulthood. It is plausible that PNA female offspring that are susceptible to PCOS already have altered gut microbial composition just after weaning, and this may be amplified by food intake and sex steroids after puberty, alongside the development of the reproductive and metabolic phenotypes of PCOS. However, whether this alteration in the microbial community influences the development of the PCOS phenotype, or whether the development of PCOS phenotype, and especially the reproductive phenotype, which precedes the metabolic phenotype, influences the microbial community is currently unclear. Future intervention studies will be required to determine whether modulation of the composition of the gut microbiome in early life (in the prepubertal, pubertal, or adolescent periods) might prevent the development of PCOS in later life.

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DISCLOSURES

The authors have stated explicitly that there is no conflict of interest in connection with this article.

AUTHOR CONTRIBUTIONS

Akari Kusamoto designed and performed the experiments, analyzed and interpreted the data, and wrote the article. Miyuki Harada conceived the work, analyzed and interpreted the data, and wrote the article. Jerilee M. K. Azhary designed and performed the experiments. Chisato Kunitomi performed the experiments, and analyzed and interpreted the data. Emi Nose, Hiroshi Koike, and Zixin Xu performed the experiments. Yoko Urata, Tetsuaki Kaku contributed to the study design and data interpretation. Nozomi Takahashi, Osamu Wada-Hiraike, Yasushi Hirota, Kaori Koga, Tomoyuki Fujii, and Yutaka Osuga contributed to data interpretation and the revision of the article. All the authors read, commented upon, and approved the final version of the manuscript.

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