

# Effects of maternal taxane chemotherapy exposure on daughters' ovarian reserve and fertility potential

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**Objective:** To investigate the long-term effects of in utero taxane exposure on exposed daughters' ovarian reserve and reproductive potential.

**Design:** Pregnant dams were treated with a single, human-relevant animal-equivalent dose of saline, docetaxel, or paclitaxel at embryonic day 16.5. In utero-exposed daughters were aged to multiple postnatal time points for ovarian and endocrine analysis or were bred to assess fertility and fecundity. Granddaughters of treated dams were assessed also for ovarian follicle composition and atresia.

**Setting:** Laboratory study.

**Animals:** C57BL/6 mice.

**Intervention(s):** In utero exposure to saline, docetaxel, or paclitaxel.

**Main Outcome Measure(s):** Ovarian follicle composition, rates of follicle atresia, and rates of multiocyte follicles were analyzed in all exposure groups. Serum hormone levels and oocyte retrieval outcomes following ovarian hyperstimulation were also assessed. Finally, animals from all exposure groups were bred with the number of litters, pups per litter, live births, interlitter time interval, and age at the last litter analyzed.

**Result(s):** We found that docetaxel and paclitaxel exposure in utero results in ovarian toxicity later in life, significantly affecting folliculogenesis as well as increasing the rate of follicular abnormalities, including follicle atresia and multiocyte follicles. Furthermore, viability staining indicates that the ovaries of daughters exposed to taxanes in utero demonstrate a significantly higher number of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive follicles. Hormone measurements also revealed that serum follicle-stimulating hormone concentration was significantly altered in taxane-exposed daughters, with the ratio of luteinizing hormone to follicle-stimulating hormone significantly elevated, specifically after paclitaxel exposure, coincident with the inability of these animals to properly respond to ovarian stimulation. Breeding studies over the course of a year also suggest that these taxane-exposed mice are fertile, although the duration of their fertility is shortened and they produce significantly fewer litters. Finally, ovarian effects are apparent in granddaughters of mice treated with docetaxel, suggesting persistent and multigenerational effects of taxane exposure.

**Conclusion(s):** Our studies demonstrate that in utero exposure to taxane-based therapy during late gestation has a significant effect on the long-term reproductive health of exposed daughters (as well as their daughters) and will be instrumental in helping clinicians better understand which chemotherapies for maternal malignancy are least detrimental to a developing fetus. (F S Sci® 2024;5:141–53. ©2023 by American Society for Reproductive Medicine.)

**Key Words:** Ovarian reserve, fertility, fecundity, oncofertility, chemotherapy

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**A**t least 1–2 in 1000 women per year are diagnosed with cancer during pregnancy, with most cancers being breast, cervical, melanoma, and ovarian (1, 2). Notably, in countries where the median age of childbearing is increasing, incidence rates are expected to surge, and more women are at risk of being diagnosed with maternal malignancy (3).

Among the various approaches to cancer treatment, chemotherapy is commonly used in standard treatment regimens to inhibit cell proliferation and tumorigenesis to shrink tumor burden, treat metastasis, and prevent cancer recurrence. For cancer detected during pregnancy, chemotherapies are used after the first trimester on the basis of positive short-term studies that indicate normal fetal development (4–7). However, because of ethical concerns regarding clinical trials on pregnant women, very few drugs have been tested on pregnant women, and most chemotherapeutics are labeled as “category D” by the US Food and Drug Administration (8). Therefore, cancer treatment during pregnancy is based on nonpregnant women’s treatment recommendations from the National Comprehensive Cancer Network with adjustments in timing, and administration is left up to the discretion of the oncologist when the potential benefits outweigh the risks (9).

Taxanes are a class of chemotherapies that include drugs such as docetaxel, paclitaxel, and cabazitaxel. Their mechanism of action proceeds by disrupting the polymerization and depolymerization of microtubules during the M phase of the cell cycle, thereby inhibiting cell division (10, 11). Docetaxel and paclitaxel have been established as part of standard treatment regimens for patients undergoing treatments for breast, lung, and ovarian cancers (12). Since their development, taxanes have been instrumental in both early-stage and advanced-stage cancer treatment and have increased survival rates for a variety of carcinomas compared with previously standard chemotherapies (13).

Some of the most well-documented side effects of chemotherapies such as taxanes include excessive oocyte loss, ovarian atrophy, primary ovarian insufficiency, and early-onset menopause, which all pose a threat to fertility potential (14, 15). Furthermore, *in vitro* studies of docetaxel on neonatal mouse ovaries have demonstrated toxicity to the somatic cells of the ovary, altering ovarian folliculogenesis (16). Female fertility potential is determined in part by the abundance and health of the ovarian reserve, which is a pool of primordial follicles containing the female germ line (17). These follicles are composed of a quiescent oocyte surrounded by a single layer of somatic cells and represent the entire reproductive capacity of a female (18). Because this cohort is established before birth in most mammals, additional oocytes cannot be generated. Thus, the ovarian reserve is a finite and nonrenewable population of cells that decreases over time. Chemotherapy is known commonly to accelerate oocyte depletion; therefore, patients are often advised of the risk of infertility associated with beginning cancer treatment (19), and preventing the excessive loss of oocytes after cancer treatment is imperative to preserving fertility potential.

In retrospective cohort studies comparing maternal and neonatal outcomes of pregnant women treated with taxanes,

these agents appear to not compromise the health of the fetus or the mother after their administration during gestation (7, 20, 21). However, chemotherapeutics often have low molecular weights and cross the placenta (22) with the rate of taxane placental transfer estimated to be between 1.72% and 8.8% (23). There is a lack of data evaluating the effects of *in utero* exposure on the developing fetal ovary, and any negative impact on the fertility and fecundity of the child would not appear until decades later. Therefore, to determine which chemotherapeutics pose the least risk to the future fertility of the fetus, our study aimed to elucidate whether the gonadal toxicity exhibited by these anticancer agents will persist after *in utero* exposure by investigating the effects of taxane-based chemotherapy on the developing fetal ovary.

Although previous studies have begun to test the ovarian effects of taxane exposure in an *in vitro* system (16), our study utilizes an *in vivo* mouse model of pregnancy to expand on these findings. In this work, we have demonstrated for the first time that *in utero* exposure to the taxanes docetaxel and paclitaxel results in a decrease in ovarian cross-sectional area and an increase in follicle atresia, especially in primary and secondary follicles. These phenotypes coincide with apparent endocrine disruption and a dramatic effect on exposed daughters’ fecundity. Finally, docetaxel-associated ovarian toxicity is observed also in the second generation (the progeny of *in utero*-exposed daughters), demonstrating a persistent effect on ovarian health.

## MATERIALS AND METHODS

### Animals

C57BL/6 mice were obtained from the Jackson Laboratory (strain No. 000664). All animal protocols were reviewed and approved by the Brown University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (No. 22-09-0002). All animal protocols were reviewed and acknowledged by the Lifespan University Institutional Animal Care and Use Committee (No. 505422).

### Treatments and Tissue Collection

Female 6-week-old (reproductively young; sexually mature) C57BL6/J WT mice were housed with male 6-week-old C57BL6/J WT mice, and copulation was confirmed by the presence of mating plugs (with this appearance considered embryonic day 0.5 [E0.5]). Pregnant mice were administered one dose (100  $\mu$ L) of saline, a matched volume of appropriately dosed docetaxel (McKesson), or paclitaxel (McKesson) using intraperitoneal injection on embryonic day 16.5 (E16.5). The dosages of docetaxel and paclitaxel were calculated to match breast cancer treatment dosages in human patients (100 mg/m<sup>2</sup> and 175 mg/m<sup>2</sup>, respectively) (24–26). Female pups resulting from the timed matings were collected at 14 days ( $n = 12$  saline,  $n = 6$  docetaxel,  $n = 6$  paclitaxel) and 30 days ( $n = 12$  saline,  $n = 9$  docetaxel,  $n = 6$  paclitaxel) after birth and killed using carbon dioxide inhalation.

The ovaries were removed, cleaned of excess fat and bursal sacs, and fixed in a 1:10 formalin solution overnight.

Afterward, ovaries were suspended in 30%, 50%, and lastly, 70% ethanol for 30 minutes each and then embedded in Histo-Gel. Standard paraffin embedding and 5  $\mu\text{m}$  serial sectioning were performed using the Brown University Molecular Pathology Core. Sections were deparaffinized using the standard protocols (27) with minor modifications: 3 15-minute washes in HistoClear followed by rehydration in 100% ethanol 2 times for 5 minutes each, 95% ethanol 2 times for 5 minutes each, 70% ethanol for 5 minutes, and water for 5 minutes. Slides were stained with either hematoxylin and eosin or with the Roche In Situ Cell Death Detection Fluorescein kit.

### Hematoxylin and Eosin Staining

Slides were dipped in hematoxylin for 2 minutes, deionized water 2 times for 10 dunks each, and bluing reagent for 4 dunks, followed by deionized water 2 times for 10 dunks each. Then, slides were immersed in eosin for 2 minutes and quickly dipped in deionized water for four half-dunks. Slides were then dehydrated in 70% ethanol (2 dunks), 80% ethanol (2 dunks), 95% ethanol 2 times for 5 minutes, 100% ethanol three times for 5 minutes, and HistoClear three times for 5 minutes, before being mounted in Permount with coverslips. Stained slides were visualized on an EVOS M5000 Fluorescence Imaging System, and images of all fields of a single section were captured at  $\times 10$  and  $\times 20$  magnifications.

### Follicle Counts

Ovarian follicle counts were quantified using two sections on every fourth slide of sectioned ovary to capture all follicle stages without overcounting of larger follicles (given 8 sections per slide, at 5  $\mu\text{m}$  per section, every fourth slide represents 160  $\mu\text{m}$  of distance). Follicle counts were normalized to section area to account for size differences between different ovaries. Follicles were classified using standard protocols (27). Follicles were counted when the oocyte cytoplasm was present. Primordial follicles were defined by one layer of flattened granulosa cells surrounding the oocyte; primary follicles were defined by a single layer of cuboidal granulosa cells surrounding the oocyte; secondary follicles were defined by two or more layers of cuboidal granulosa cells surrounding the oocyte; and preantral follicles were classified as follicles in which antral space had begun to form among the granulosa cells. Follicles were classified as atretic when they contained a degenerating oocyte, indicated by the presence of vacuous cytoplasmic space, visible blebbing, or collapse of the somatic cell interface. All ovarian sections used for follicle quantification for a given sample were also analyzed for the ovarian area, with the areas of each section averaged within an animal and then within a treatment group.

### TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE dUTP NICK END LABELING (TUNEL) STAINING

Slides were stained with the Roche In Situ Cell Death Detection Fluorescein kit per the manufacturer's instructions. Briefly, deparaffinized slides were washed in phosphate-buffered saline (PBS) for 3 minutes and then covered with 200  $\mu\text{L}$  of 20  $\mu\text{g}/$

mL proteinase K in 10 mM Tris-HCl to expose binding sites. Slides were then incubated in a humid chamber for 15 minutes at room temperature. Afterward, slides were washed two times for 3 minutes each in PBS, followed by a 3-minute wash in PBS with Tween 80, and two times for 3 minutes each in PBS. A total of 100  $\mu\text{L}$  of 90  $\mu\text{L}$  TUNEL labeling solution and 10  $\mu\text{L}$  TUNEL enzyme were added to each slide and incubated at 37 °C for 1 hour. Slides were washed with PBS three times for 3 minutes each, once for 3 minutes with a mixture of 100 mL PBS and 10  $\mu\text{L}$  of 10 mg/mL 4',6-diamidino-2-phenylindole stock, and once for 5 minutes with PBS. Finally, coverslips were mounted with antifade 4',6-diamidino-2-phenylindole mounting media. Slides were visualized and analyzed for TUNEL-positive follicles, which were defined as any follicle containing any positive oocyte or somatic cells.

### Serum Hormone Analysis

A total of 20 ( $n = 5$  saline,  $n = 7$  docetaxel,  $n = 8$  paclitaxel) 30-day-old mice were exsanguinated with whole blood collected using cardiac puncture. Blood was collected into serum separator tubes, allowed to clot for 30 minutes, and then spun at  $3,000 \times g$  for 15 minutes at 4 °C. The supernatant was collected and stored at  $-80$  °C. Serum was sent to the University of Virginia Ligand Assay and Analysis Core of the Center for Research in Reproduction for analysis of anti-müllerian hormone (AMH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) serum concentrations.

### Ovarian Hyperstimulation

Mice ( $n = 11$  saline,  $n = 8$  docetaxel,  $n = 11$  paclitaxel) at 1 month of age were hormonally stimulated using standard protocols (28). Briefly, mice were injected intraperitoneally with 5-IU pregnant mare goat serum. Forty-eight hours later, the same mice were intraperitoneally injected with 5-IU human chorionic gonadotropin. Twelve hours later, mice were killed, and ovaries and oviducts were collected. Ovulated cumulus-oocyte complexes were released from the ampulla of the oviduct and counted per mouse. The number of oocytes per mouse was averaged between genotypes and compared.

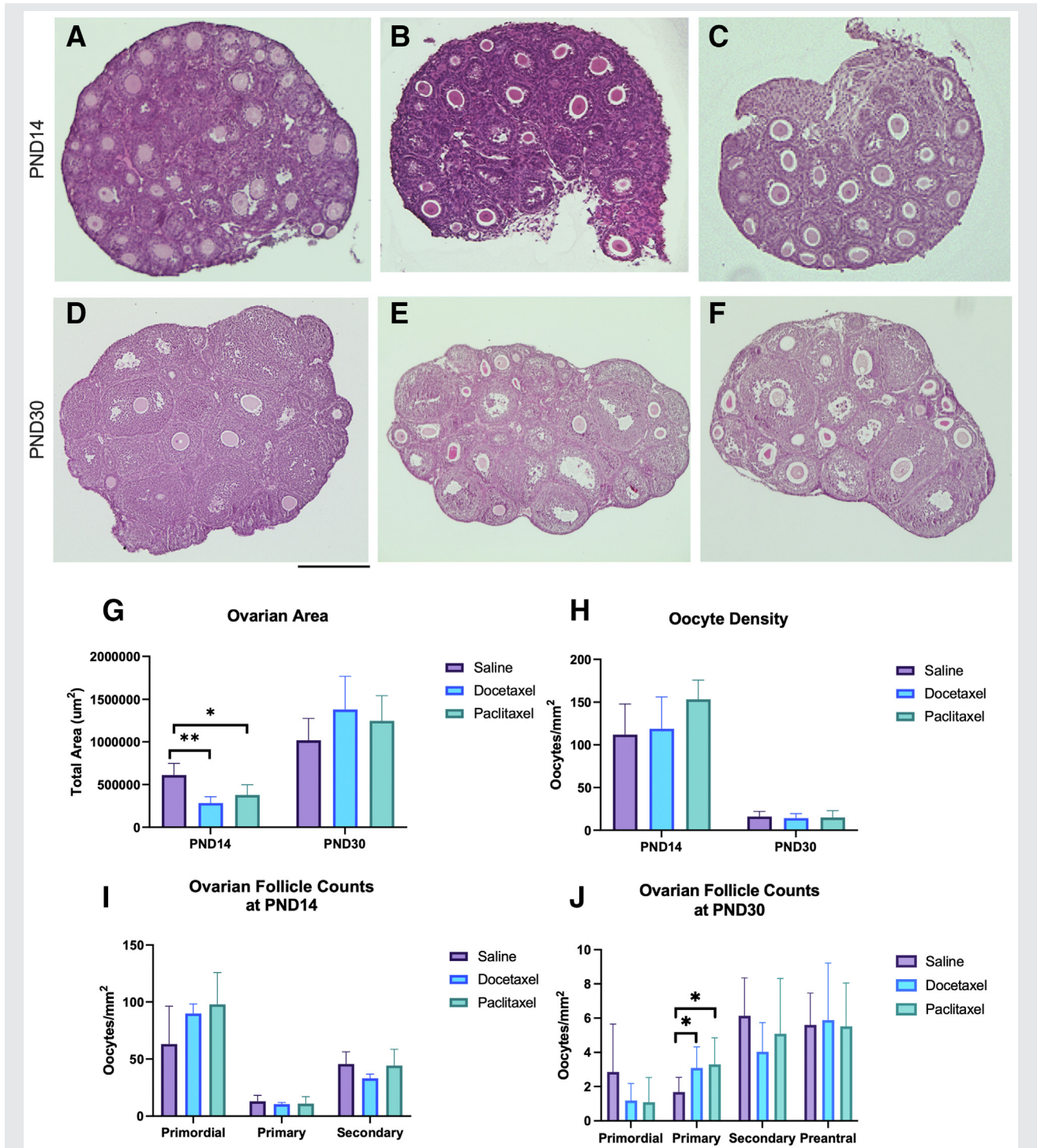
### Fertility Trials

To assess female fertility, controlled fertility trials were performed. Female mice exposed in utero to saline ( $n = 4$ ), docetaxel ( $n = 4$ ), or paclitaxel ( $n = 4$ ) were housed with wild-type C57BL/6 males, both at 6 weeks of age. All animals were housed in single breeding pairs with love huts and monitored for signs of copulation, pregnancy, and the birth of pups. The number of pups per litter and the number of survived pups were recorded over time. All breedings were discontinued after 1 year. The resulting female ( $n = 5$  saline,  $n = 5$  docetaxel,  $n = 5$  paclitaxel) pups (the "F2 generation") from these breedings were collected at 14 days of age for multigenerational ovarian analysis.

### Statistical Analysis

All statistical analyses were performed in GraphPad Prism, and all data were assessed for normal distribution. Parametric

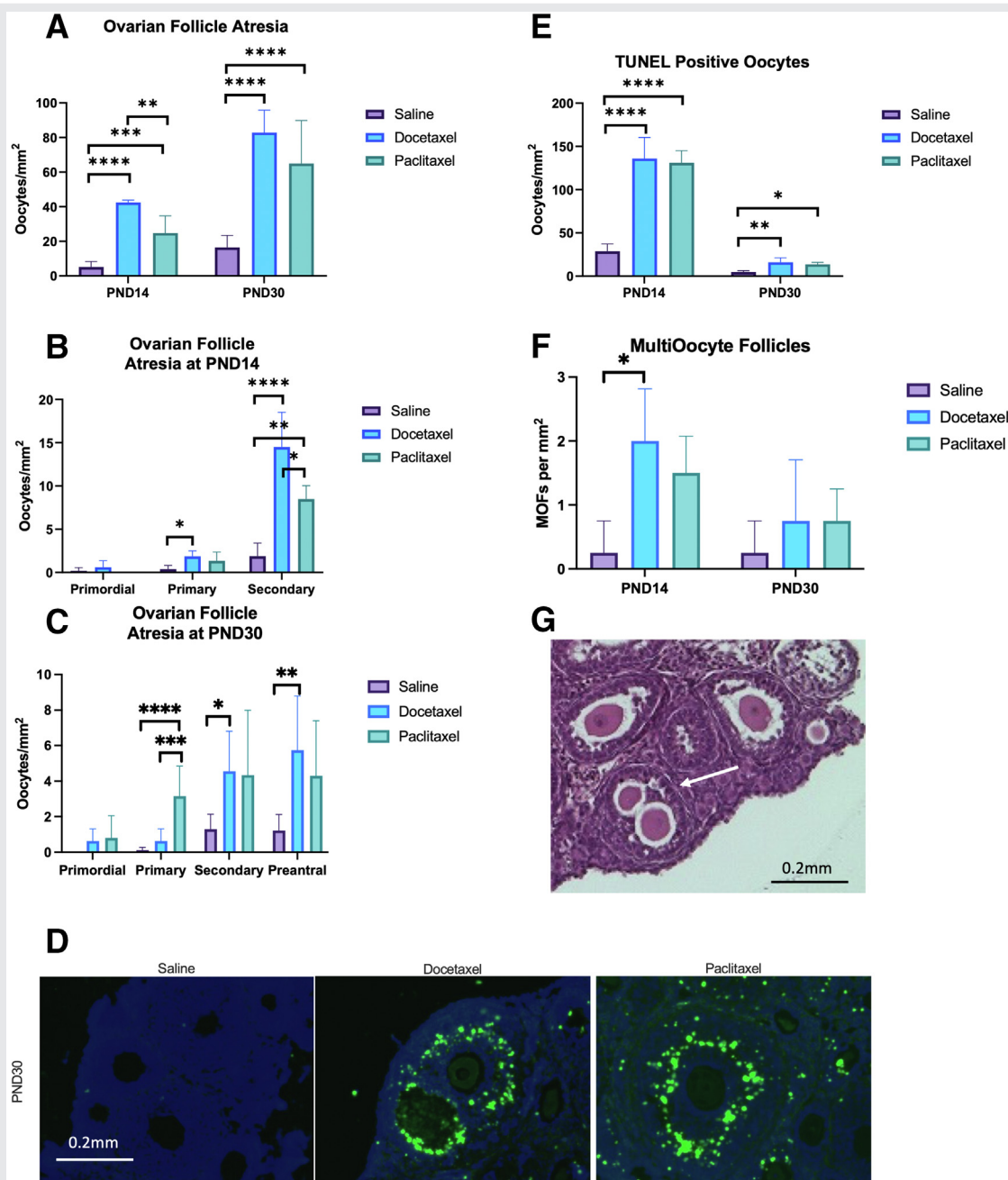
**FIGURE 1**



In utero taxane exposure disrupts post-natal folliculogenesis. PND14 (A-C) and PND 30 (D-F) ovaries from control, Docetaxel-, and Paclitaxel-exposed female mice were collected, paraffin-embedded, and stained by standard hematoxylin and eosin protocols. White arrows denote degenerating follicles. (G) Ovarian size is significantly reduced in both taxane exposed groups at PND14, while overall oocyte density is unchanged at either time point (H). (I, J) Ovarian follicle counts were quantified using two sections on every fourth slide of sectioned ovary to capture all follicle stages without over-counting of larger follicles. Follicle counts were normalized to the section area to account for size differences between different ovaries. Overall follicle composition is unchanged between treatments groups with the exception of increased primary follicles in both taxane-exposed groups at PND30.

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FIGURE 2



In utero taxane exposure increase follicular abnormalities and atresia. (A) Follicle atresia was identified by vacuous cytoplasmic space, visible blebbing, or collapse of the basement membrane. Both treatment groups have greater incidence of follicle atresia at PND14 and PND30 compared to controls. (B, C) Docetaxel-exposed animals possess increased atretic primary and secondary follicles at PND14 as well as PND30, as well as increased atretic preantral follicles at PND30, while paclitaxel-exposed animals possess increase atretic secondary follicles at PND14 and increased primary follicles at PND30. (D) TUNEL staining (green) of exposed PND30 ovaries demonstrates increased apoptotic follicles in both taxane exposed groups compared to control ovaries (all nuclei are stained in blue by DAPI). (E) Quantification of TUNEL staining demonstrates a significant increase in TUNEL-positive follicles in both taxane-exposed groups at PND14 and PND30. (F, G) MultiOocyte follicles, defined by the presence of two or more oocytes within a single basement membrane as observed at significantly greater levels in Docetaxel-exposed ovaries at PND14, with a non-significant trend toward the same phenotype in Paclitaxel-exposed animals.

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data were analyzed using a one-way analysis of variance with Tukey's multiple comparisons test applied. Differences were considered statistically significant when adjusted  $P$  values were  $P < .05$ .

## RESULTS

### Taxane Exposure in utero Does Not Affect postnatal Oocyte Density but Does Affect Ovarian Size and Folliculogenesis

To assess the effects of in utero taxane exposure on long-term ovarian health, we treated pregnant dams with a single animal-equivalent dose of docetaxel or paclitaxel, or an equal volume of control saline, at E16.5 (approximately 4 days before birth). This murine time point was chosen to model second to third-trimester human fetal ovary development (17) and the time at which chemotherapy would be most commonly administered in the case of maternal malignancy (5, 6). Because taxane therapy is typically administered every 1–3 weeks in the human patient population (24, 25), a single dose administered to pregnant dams before birth is an appropriate analog for this schedule. Exposed and control pups ("F1 generation" pups) were then allowed to age until postnatal day (PND) 14, or PND30, followed by ovary collection to assess juvenile and prepubertal ovarian health (Fig. 1A to F). Overall oocyte number and follicle substages were then quantified and normalized to the ovary area to account for size differences between ovaries.

At both PND14 and PND30, overall oocyte density is unchanged between the control and exposure groups, despite a significant alteration in ovarian folliculogenesis in both exposure groups. Before sexual maturity at PND14, no significant differences in folliculogenesis are observed despite significantly smaller ovaries in both docetaxel- and paclitaxel-exposed daughters (Fig. 1G to I,  $P = .0024$  and  $.0121$ , respectively). Two weeks later, however, at PND30, both exposure groups possess an increased density of primary follicles (Fig. 1J,  $P = .0500$  and  $.0497$ , respectively) and a commensurate nonsignificant decrease in primordial and secondary follicle density. These results demonstrate that although overall oocyte abundance remains unchanged, at least at these juvenile and early adult time points, the dynamics of folliculogenesis are considerably altered after a single in utero exposure to taxane-based chemotherapy.

### In utero Taxane Exposure Increases the Incidence of Follicular Abnormalities and Atresia

In addition to altered follicle composition in taxane-exposed daughters, the number of atretic and degenerating follicles was also significantly increased. Docetaxel-exposed daughters have a far greater incidence of ovarian follicle atresia at PND14 as well as PND30 ( $P < .0001$  at both time points), which is also observed in paclitaxel-exposed daughters ( $P < .001$  at PND14 and  $P < .0001$  at PND30) (Fig. 2A). Despite this increase in follicle atresia at PND14, paclitaxel appears significantly less toxic than docetaxel at this time point ( $P = .0026$ ). When the substages of the degenerating follicles were analyzed, the effects were seen primarily in activated

and maturing follicles, not in the resting primordial follicle pool. At PND14, docetaxel-exposed daughters possess significantly more degenerating primary follicles than saline controls ( $P = .0333$ ), although both docetaxel- and paclitaxel-exposed daughters possess significantly more degenerating secondary follicles ( $P < .0001$  and  $P = .0038$ , respectively) (Fig. 2B). Again, docetaxel appears to exhibit higher toxicity than paclitaxel, with significantly more degenerating secondary follicles than their paclitaxel-exposed counterparts ( $P = .0102$ ).

Interestingly, the degeneration of primary follicles in paclitaxel-exposed daughters does not appear until PND30, when these animals possess significantly more atretic primary follicles than saline controls ( $P < .0001$ ) or docetaxel-exposed counterparts ( $P = .0001$ ). The degenerative effects of docetaxel at later follicle stages also persist at PND30, with significantly more atretic secondary ( $P = .0228$ ) and preantral ( $P = .0036$ ) follicles than saline controls (Fig. 2C). When TUNEL analysis was performed on the same ovaries, both exposure groups possessed significantly more TUNEL-positive follicles than controls at both time points ( $P < .0001$  for both docetaxel and paclitaxel at PND14;  $P = .0025$  and  $.01014$ , respectively, at PND30) (Fig. 2D and E).

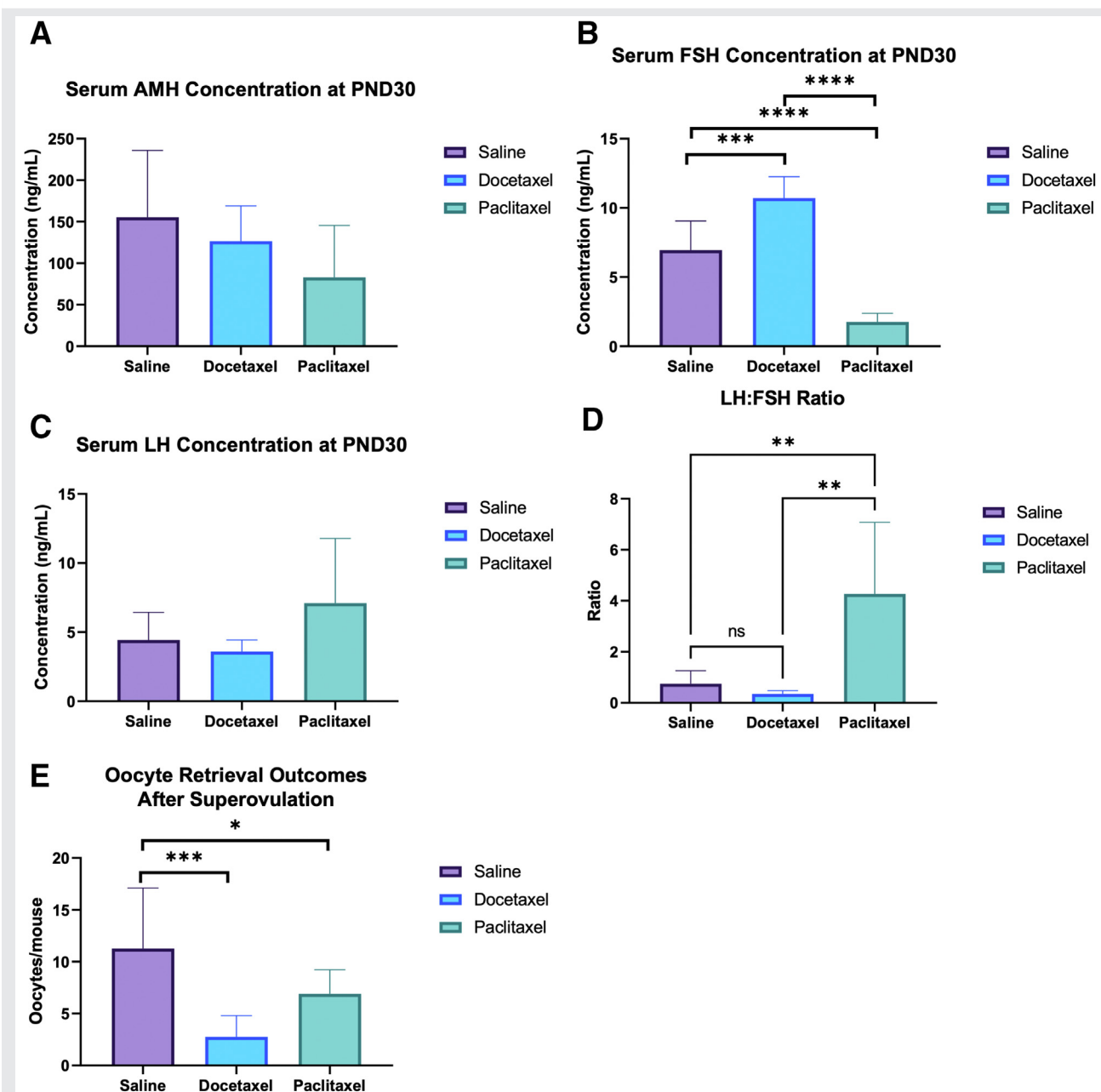
There was also an increase in the number of multioocyte follicles (MOFs) in taxane-exposed mice. Multioocyte follicles are formed when two or more oocytes are contained within a single follicle structure and basement membrane and are not typically developmentally competent because of insufficient somatic cell support for the contained oocytes (29). At PND14, docetaxel-exposed ovaries possess significantly increased densities of MOFs ( $P = .0101$ ), although paclitaxel-exposed ovaries possess a nonsignificant increase ( $P = .0543$ ) (Fig. 2F and G). In addition, this effect is observed, although not significant, at PND30.

### In utero, Taxane Exposure Results in Altered Gonadotropin Levels and Reduced Ovulatory Capacity

Serum hormone and gonadotropin levels were also profiled because the steroid hormones produced by the ovary participate in and influence the function of the hypothalamic-pituitary-gonadal (HPG) axis. Serum was purified from control and exposed daughters at 1 month of age, with levels of AMH, FSH, and LH profiled. Antimüllerian hormone levels, a proxy for follicle abundance (30), were unchanged in the exposure groups (Fig. 3A), which is consistent with the finding that overall oocyte abundance is also unchanged in these ovaries, with LH levels were also unchanged (Fig. 3C). Interestingly, however, FSH levels were significantly increased in the serum of docetaxel-exposed daughters ( $P < .0001$ ), although paclitaxel-exposed daughters possess significantly decreased FSH levels ( $P < .001$ ) (Fig. 3B). When these FSH levels were compared with LH levels, however, only paclitaxel-exposed animals exhibited an altered ratio of LH to FSH, which was significantly elevated above that of controls or docetaxel-exposed animals (Fig. 3D).

Superovulation was performed to investigate the functional status of the ovarian reserve and HPG axis. Both

FIGURE 3



In utero taxane exposure results in endocrine disruption and ovulatory capacity. (A) Serum AMH is not significantly different among the treatment groups at PND30. (B) Serum FSH levels are significantly altered in both treatment groups at PND30, while serum LH levels are unchanged (C). (D) LH:FSH ratios for all treatment groups. (E) At 1 month of age, animals of all exposures were super-ovulated using standard pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) protocols. Retrieved oocytes were collected from each animal, counted, and averaged per treatment group. Both taxane-exposed groups ovulate significantly fewer oocytes following ovarian stimulation.

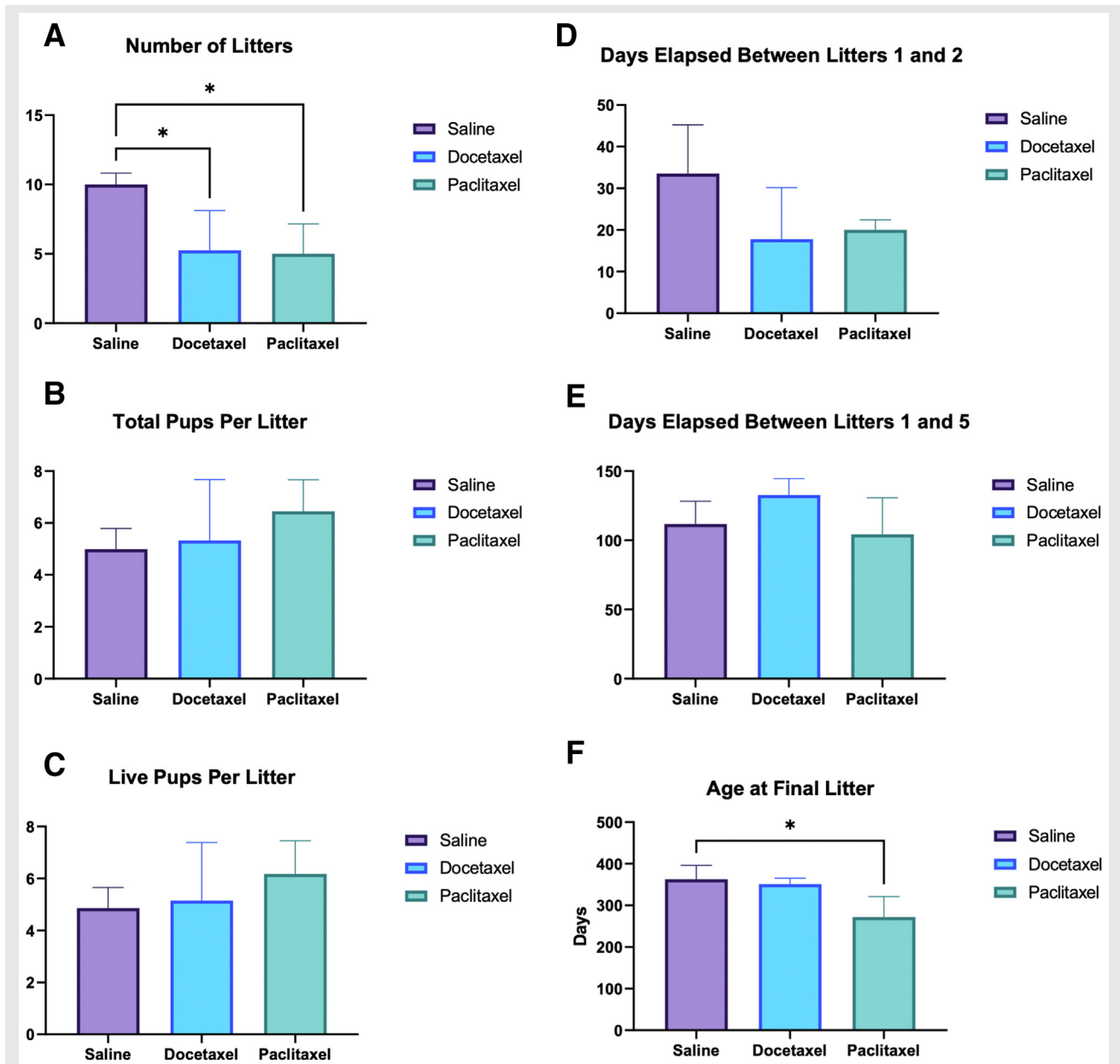
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docetaxel- and paclitaxel-exposed daughters ovulate significantly fewer oocytes than control-treated mice ( $P=.003$  and  $.0490$ , respectively) (Fig. 3E), which is consistent with the hypothesis that in utero taxane exposure may result in delayed or inhibited ovarian folliculogenesis, including in response to hormonal hyperstimulation.

### In utero, Taxane Exposure Affects Long-Term Fertility and Fecundity of Exposed Daughters

To determine the long-term reproductive effects of in utero taxane exposure, additional control and treated animals were paired with verified C57BL/6J male breeders in single

## FIGURE 4



In utero taxane exposure reduces long-term fertility and fecundity. (A, B) Number of litters and pups per litter were graphed for all breeders in the three in utero exposure groups, demonstrating normal litter sizes but reduced overall litters in both taxane-exposed groups. (C) Number of live births is unchanged in any exposure group. (D, E) Inter-litter intervals are unchanged between exposure groups. (F) Paclitaxel-exposed animals have a significantly shorter reproductive lifespan than control animals.

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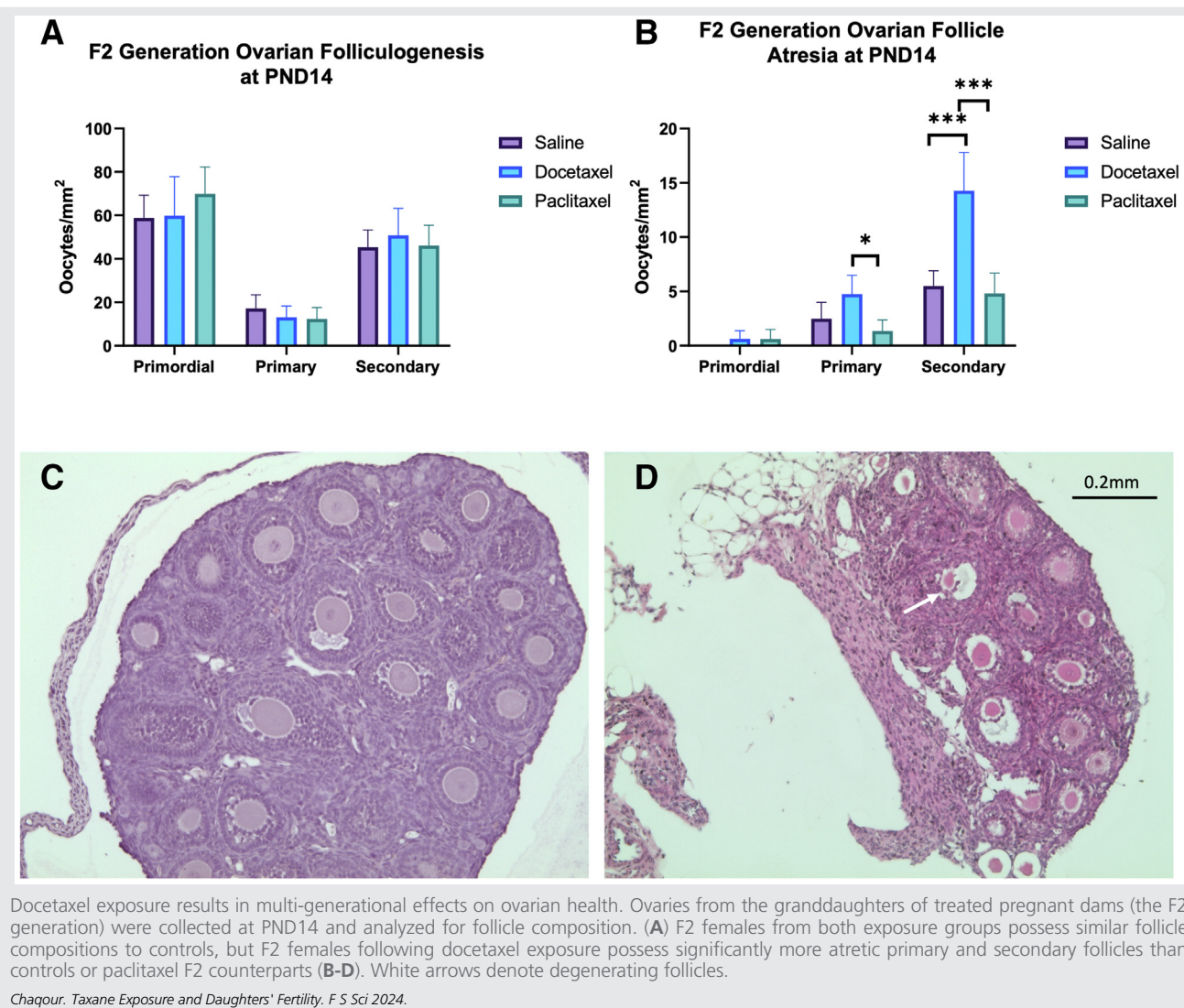
breeding cages at 6 weeks of age. Mice were observed until 1 year of age, during which time the number of litters, pups per litter, interlitter intervals, and the number of deceased pups were recorded. Although the litter sizes, interlitter intervals, and number of live pups were unchanged between groups (Fig. 4 to E), both docetaxel- and paclitaxel-exposed daughters produce significantly fewer litters than age-matched control females ( $P=.0283$  and  $.0219$ , respectively) (Fig. 4A). Interestingly, paclitaxel-exposed daughters also deliver their

last litter earlier in the reproductive lifespan than control females (271.7 vs. 362.8 days, SE = 26.81,  $P=.0272$ ), suggesting a premature reproductive decline (Fig. 4F).

### In utero, Docetaxel Exposure Results in Multigenerational Effects on Ovarian Function

Finally, to assess the potential transgenerational effects of taxane exposure, female pups from our breeding trials

## FIGURE 5



(granddaughters of the dams treated with taxane-based chemotherapy during pregnancy; “F2 generation” pups) were killed at PND14 and their ovaries analyzed for follicle composition. Although folliculogenesis appears unaffected (Fig. 5A), (as expected given that these animals were not directly exposed to any taxanes but were instead a product of oocytes that were exposed in utero), a significant increase in primary and secondary follicle degeneration was observed in docetaxel F2 females compared with control F2 females ( $P=.0110$  and  $< .001$ , respectively) (Fig. 5B to D). These trans-generational effects on follicle degeneration were not observed in paclitaxel F2 females.

## DISCUSSION

The ovarian reserve represents the entire fertility potential of an individual, and preserving its health and quantity is of

utmost importance. Because of the well-documented detrimental effects of chemotherapies on fertility, it is important to extend the research to studying the ability of chemotherapies to affect the child in utero, as the incidence rate of maternal malignancy increases each year as more women delay childbirth. In this study, we characterized the effects of two taxane chemotherapies, docetaxel, and paclitaxel, on the developing fetal ovary via in vivo studies analyzing two separate time points, PND14 and PND30, which represent two key time periods before sexual maturity.

In this work, we have found that there is a stark difference between the quality and quantity of oocytes after in utero exposure. Interestingly, oocyte density remains intact and unchanged across treatment groups (Fig. 1H), despite the notable appearance of degenerative follicles in the taxane-exposed ovaries (Fig. 2A). This finding was somewhat unexpected because one might expect to see a diminished ovarian

reserve after exposure to chemotherapy, as seen in many previous studies that have shown loss of primordial follicles and ovarian atrophy (31). Instead, on analysis of oocyte density per stage at PND14, the amounts of primordial follicles across the groups are relatively similar and nonsignificant, and the only difference appears in the secondary stage, where docetaxel-exposed ovaries exhibit a nonsignificantly reduced density (Fig. 1I). At PND30, the taxane-exposed ovaries have a nonsignificant decrease in primordial follicle density, although they also have a significantly higher primary follicle density (Fig. 1J). At the secondary stage, again there is a nonsignificant decrease in secondary oocyte density for both exposure groups, recapitulating the same finding from PND14. One possible explanation for this pattern of folliculogenesis is that chemotherapy may be inhibiting follicle maturation at the primary to secondary stage, which would be consistent with previous *in vitro* studies of docetaxel-associated ovarian toxicity showing that it is damaging to granulosa cells of growing follicles with no effects on primordial follicles or oocytes themselves, which results in docetaxel effects on the ovary (16). Taxane exposure may also induce premature activation of primordial follicles, as has been demonstrated for other cytotoxic chemotherapies (32, 33), with this activation potentially secondary to loss of inhibition. In the event of toxicity to, and loss of, immature growing follicles, reduced AMH secretion may potentially release follicles from inhibition (34), allowing for a wave of premature activation and reduced primordial follicles overall, with an ultimate stabilization of AMH levels later in life; further studies will be necessary to conclusively demonstrate the mechanism underlying this phenotype.

In addition, we analyzed ovarian area per section and found a significant decrease in the sizes of taxane-exposed ovaries compared with the control, however, this finding did not persist past the PND14 time point (Fig. 1G) and may reflect specific effects on somatic cell expansion before pubertal onset coupled with a greater proportion of primordial follicles in these animals (Fig. 1I), although this finding did not reach statistical significance. Similarly, the presence of multioocyte follicles, a prominent follicular abnormality, was only significantly heightened at PND14 and not PND30 in taxane-exposed ovaries (Fig. 2F and G). However, the presence of MOFs indicates that folliculogenesis may be impaired, which is further concerning because research suggests that MOFs have a 30% lower fertilization rate than their monoovular counterparts and are also less viable (35, 36).

Most significantly, follicle atresia is significantly increased overall in both docetaxel- and paclitaxel-exposed ovaries (Fig. 2A). Specifically, atresia is seen to be significantly heightened in the primary and secondary stages at PND14 after docetaxel exposure and secondary-only stages after paclitaxel exposure (Fig. 2B); however, a more drug-specific pattern is seen at PND30. Here, only docetaxel-exposed ovaries have significantly higher average degenerating follicles per section at the secondary and preantral stages, although the paclitaxel group demonstrates significantly higher atresia only at the primary stage (Fig. 2C). Overall, these results, combined with the stage-specific oocyte densities, suggest that the precise dynamics of taxane-induced

ovarian toxicity are different between the two agents, with docetaxel affecting all follicle stages through preantral, whereas paclitaxel-induced toxicity appears restricted to earlier stage follicles.

The mechanism by which chemotherapy disturbs the ovarian reserve is most commonly thought to be oocyte death by apoptosis (31, 37) because most chemotherapeutic agents act by damaging DNA during the S phase of mitosis. Here, we have demonstrated a significantly higher number of TUNEL-positive follicles in taxane-treated ovaries than in the control (Fig. 2D and E), although we cannot determine from these data whether it is the apoptosis of follicular cells causing oocyte damage despite no obvious TUNEL staining of the oocyte itself or whether it is a poor-quality oocyte resulting in follicular failure and somatic cell apoptosis. Previous research demonstrates that when DNA damage occurs, an oocyte attempts to repair this damage to the DNA via the ataxia-telangiectasia mutated-mediated repair pathway (38). When repair is not an option for severely damaged oocytes, apoptosis is triggered, causing a potential reduction in the ovarian reserve. This repair mechanism is important in maintaining fertility and preserving the abundance of oocytes in the ovary and may explain why paclitaxel-exposed follicles demonstrate altered oocyte morphology at the primary stage of PND30 but go on to further develop into secondary and preantral follicles at no cost to oocyte density. These damaged oocytes may be capable of functionally efficient DNA repair that allows for oocyte survival.

Another indicator of ovarian health and HPG axis function is the serum concentration of steroid hormones and gonadotropins. AMH is commonly understood to be a hormone reflective of the abundance of activated follicles in the ovarian reserve, and the growth of small follicles (30). We found a nonsignificant decrease in AMH levels between the control and taxane-exposed mice at PND30 (Fig. 3A), which is consistent with the nonsignificant difference in oocyte densities across the treatment groups at both time points. Follicle-stimulating hormone and LH are secreted from the anterior pituitary and are important for maintaining ovulatory function (39). Variations in FSH and LH levels of secretion are because of the feedback mechanisms that exist between the ovary, pituitary, and hypothalamus. Indeed, abnormal levels of FSH and LH have been shown to be characteristic of women with a decreased ability to conceive (40). In our study, we show that docetaxel-exposed mice have significantly higher levels of serum FSH concentration, which is a marker of premature ovarian failure and a manifestation of ovarian aging (41). In contrast, paclitaxel-exposed animals possessed a significantly reduced serum concentration of FSH (Fig. 3B), whereas levels of serum LH concentration remained relatively unchanged (Fig. 3C). When compared, the ratio of LH to FSH was perturbed only in paclitaxel-exposed animals, with significant elevation over control and docetaxel-exposed counterparts. Although the mechanisms for these phenotypes cannot be conclusively demonstrated from these data, the pathogenesis of this paclitaxel-induced toxicity may be related to altered gonadotropin hormone-releasing hormone (GnRH) pulsatility resulting from gestational exposure. Indeed, it has been demonstrated previously that taxanes can cause toxicity to

the hypothalamus (42), which is likely to result in altered release of GnRH. In mice, frequent and higher amplitude GnRH pulses from the hypothalamus favor the release of LH from the pituitary, although slower pulses favor the release of FSH (43). Therefore, in the case of paclitaxel-exposed animals, GnRH pulsatility may be affected, causing a relative increase in LH levels compared with FSH levels. Importantly, serum hormone levels were obtained from animals before sexual maturity, so these results may be also reflective of a differential timeline of pubertal onset in paclitaxel-exposed animals compared with docetaxel-exposed or controls because elevated LH levels in the presence of normal FSH levels in prepubertal girls have been associated with central precocious puberty (44, 45). Future studies are required to disentangle these potential mechanisms, including the ways in which steroid hormone levels, such as estradiol, may be also affected and involved in altered gonadotropin levels (46). Moreover, ovarian stimulation of the taxane-exposed daughters at 1 month of age demonstrated severely compromised ovulatory capacity because the number of retrieved oocytes was significantly lower than the control group (Fig. 3E). Further studies will be required to determine the mechanism of this ovulatory dysregulation and the ability of these animals to ovulate at other time points.

Still, despite concerns that these mice are unable to ovulate efficiently, our breeding studies, which took place over the course of a year, indicate that mice exposed to taxanes in utero are, in fact, fertile. However, analysis shows that they produce a significantly fewer number of litters (Fig. 4A), although the number of pups per litter remains unchanged (Fig. 4B). There is also no evidence that the rate of stillbirth has increased, as the number of live births remains similar to the control (Fig. 4C). Interestingly, we did observe premature reproductive senescence specifically in the paclitaxel-exposed group, with a significantly earlier age at the final litter (Fig. 4F).

Finally, we also observed a surprising transgenerational effect of docetaxel exposure on the folliculogenesis of the F2 generation. When the ovaries of these animals were histologically analyzed for follicle composition, no significant differences were observed (Fig. 5A). However, there were significantly more degenerating secondary follicles in the F2 generation after docetaxel exposure of their grandmothers compared with control F2 females or the F2 generation after paclitaxel exposure (Fig. 5B to D). Although this result is surprising, it is not without precedent. Other studies have observed also transgenerational effects of chemotherapy exposure, both in terms of disease incidence and fecundity in the human population (47). The mechanisms for these effects are not clear, but they may be because of the persistent and heritable epigenetic alterations in the germ line that affect oocyte quality (48).

It is intriguing to observe the nuanced differences between docetaxel and paclitaxel exposure, despite shared general mechanisms of action. Although both agents target microtubule stability, there are demonstrated differences in the exact biochemical mechanisms of these two agents, with docetaxel exhibiting greater ability to bind to  $\beta$ -tubulin and inhibit microtubule depolymerization, whereas paclitaxel specifically affects the organization of mitotic spindles

(49, 50). In general, we have found that docetaxel-associated ovarian toxicity is more severe and pervasive, as well as persistent in the F2 generation. Furthermore, when FSH levels are altered in both exposure groups, docetaxel-exposed daughters possess significantly elevated serum FSH levels, although paclitaxel-exposed daughters' serum FSH levels are significantly reduced compared with controls, suggesting that the downstream neuroendocrine effects of these taxanes may differ. Although elevated FSH levels are known to be associated with premature ovarian aging, the reduced FSH levels and elevated ratio of LH to FSH after paclitaxel exposure may be because of broader toxicity to the HPG axis (41–43). Given these endocrine effects, it is also critical to note that although many of the phenotypes observed in exposed animals are ovary-specific, particularly follicular atresia and apoptosis, the effects of taxane exposure may also be indirect or secondary, via additional neuroendocrine mechanisms. Further research will be critical in determining whether the folliculogenesis and fertility effects of in utero exposure to taxanes are because of direct damage to the ovary or a more generalized toxicity to the HPG axis.

A strength of this study is the clear in vivo demonstration that taxanes affect the ovarian reserve of the developing fetal ovary in a murine model, likely because of their ability to cross the placenta (23, 51–53). Still, despite these findings, more research is needed to properly elucidate other possible ways in which the ovary is receiving damage as well as the mechanism by which this damage can be repaired. Further, this study has shed light on the detrimental effects of taxanes in a mouse model, a class of chemotherapeutics previously thought not to disturb fetal development. Thus, it raises questions about the similar gonadotoxic effects of other chemotherapeutics administered during pregnancy, as well as the combined effects of different drugs as cancer treatment regimens become more robust and complex. Although one potential strategy for mitigating chemotherapy-induced toxicity to a developing fetus is to deliver these children before their term, this approach is not favored because preterm delivery has been associated with more severe developmental complications than the treatment itself (54–56). Longitudinal data after exposed human offspring beyond initial gestation, birth, and the first few years of life are sorely needed.

## CONCLUSION

Our studies demonstrate that in utero exposure to taxane-based therapy during late gestation has a significant effect on the long-term reproductive health of exposed daughters (as well as their daughters). Through this research, we have been able to gain a more comprehensive understanding of chemotherapeutic exposure to the developing fetal ovary and aid us in our overarching goal of determining which chemotherapeutics are least detrimental for use in maternal malignancy.

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