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ORIGINAL ARTICLE *Reproductive biology*

The PARP inhibitor, olaparib, depletes the ovarian reserve in mice: implications for fertility preservation

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STUDY QUESTION: What is the impact of the poly(ADP-ribose) polymerase (PARP) inhibitor, olaparib, alone or in combination with chemotherapy on the ovary in mice?

SUMMARY ANSWER: Olaparib treatment, when administered alone, depletes primordial follicle oocytes, but olaparib does not exacerbate chemotherapy-mediated ovarian follicle loss in mice.

WHAT IS KNOWN ALREADY: The ovary contains a finite number of oocytes stored within primordial follicles, which give rise to all mature ovulatory oocytes. Unfortunately, they are highly sensitive to exogenous DNA damaging insults, such as cytotoxic cancer treatments. Members of the PARP family of enzymes are central to the repair of single-strand DNA breaks. PARP inhibitors have shown promising clinical efficacy in reducing tumour burden, by blocking DNA repair capacity. Olaparib is a PARP1/2 inhibitor recently FDA-approved for treatment of *BRCA1* and *BRCA2* mutation carriers with metastatic breast cancer. It is currently being investigated as an adjunct to standard treatment at an earlier stage, potentially curable, *BRCA1*- and *BRCA2*-associated breast cancer which affects reproductive age women. Despite this, there is no preclinical or clinical information regarding the potential impacts of olaparib on the ovary or on female fertility. Unfortunately, it may be many years before clinical data on fertility outcomes for women treated with PARP inhibitors becomes available, highlighting the importance of rigorous preclinical research using animal models to establish the potential for new cancer therapies to affect the ovary in humans. We aimed to comprehensively determine the impact of olaparib alone, or following chemotherapy, on the ovary in mice.

STUDY DESIGN, SIZE, DURATION: On Day 0, mice ($n = 5$/treatment group) were administered a single intraperitoneal dose of cyclophosphamide (75 mg/kg/body weight), doxorubicin (10 mg/kg), carboplatin (80 mg/kg), paclitaxel (7.5 mg/kg) or vehicle control. From Days 1 to 28, mice were administered subcutaneous olaparib (50 mg/kg) or vehicle control. This regimen is proven to reduce tumour burden in preclinical mouse studies and is also physiologically relevant for women.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Adult female wild-type C57BL6/J mice at peak fertility (8 weeks) were administered a single intraperitoneal dose of chemotherapy, or vehicle, then either subcutaneous olaparib or vehicle for 28 days. Vaginal smears were performed on each animal for 14 consecutive days from Days 15 to 28 to monitor oestrous cycling. At 24 h after final treatment, ovaries were harvested for follicle enumeration and immunohistochemical analysis of primordial follicle remnants (FOXL2 expressing granulosa cells), DNA damage (γH2AX) and analysis of apoptosis by TUNEL assay. Serum was collected to measure circulating anti-Müllerian hormone (AMH) concentrations by ELISA.

MAIN RESULTS AND THE ROLE OF CHANCE: Olaparib significantly depleted primordial follicles by 36% compared to the control ($P < 0.05$) but had no impact on other follicle classes, serum AMH, corpora lutea number (indicative of ovulation) or oestrous cycling.

Primordial follicle remnants were rarely detected in control ovaries but were significantly elevated in ovaries from mice treated with olaparib alone ($P < 0.05$). Similarly, DNA damage denoted by γH2AX foci was completely undetectable in primordial follicles of control animals but was observed in ~10% of surviving primordial follicle oocytes in mice treated with olaparib alone. These observations suggest that functional PARPs are essential for primordial follicle oocyte maintenance and survival. Olaparib did not exacerbate chemotherapy-mediated follicle depletion in the wild-type mouse ovary.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: This study was performed in mice, so the findings may not translate to women and further studies utilizing human ovarian tissue and sera samples should be performed in the future. Only one long-term time point was analysed, therefore olaparib-mediated follicle damage should be assessed at more immediate time points in the future to support our mechanistic findings.

WIDER IMPLICATIONS OF THE FINDINGS: Olaparib dramatically depleted primordial follicles and this could be attributed to loss of intrinsic PARP-mediated DNA repair mechanisms. Importantly, diminished ovarian reserve can result in premature ovarian insufficiency and infertility. Notably, the extent of follicle depletion might be enhanced in *BRCA1* and *BRCA2* mutation carriers, and this is the subject of current investigations. Together, our data suggest that fertility preservation options should be considered for young women prior to olaparib treatment, and that human studies of this issue should be prioritized.

STUDY FUNDING/COMPETING INTEREST(S): This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS. This work was supported by funding from the National Health and Medical Research Council (NHMRC); (K.J.H. #1050130) (A.L.W. #1120300). K.A.P. is a National Breast Cancer Foundation Fellow (Australia—PRAC-17-004). K.A.P. is the Breast Cancer Trials (Australia) Study Chair for the OlympiA clinical trial sponsored by AstraZeneca, the manufacturer of olaparib. All other authors declare no competing financial or other interests.

Key words: primordial / oocyte / DNA repair / PARP / BRCA1 / BRCA2 / olaparib / fertility

Introduction

The immature oocytes stored in primordial follicles in the ovary are the storage unit of the female germline and are non-renewable. All mature, hormone-producing oocytes are drawn from the pool of primordial follicles (McGee and Hsueh, 2000; Wallace and Kelsey, 2010). The pool diminishes with age, until the loss of fertility and onset of menopause. It is well-established that their depletion can be accelerated in response to exogenous genotoxic insults (reviewed by Morgan *et al.* (2012), Bedoschi *et al.* (2016), Winship *et al.* (2018)). Consequently, infertility and early menopause are major concerns for younger cancer survivors (Ruddy and Partridge, 2012).

Cytotoxic chemotherapy is a mainstay of cancer treatment. While effective, it is associated with systemic off-target effects, including impacts on fertility. New treatment approaches include small molecule inhibitors, developed with the purpose of having more targeted effects on cancer cells, thus reducing off-target effects. Members of the poly(ADP-ribose) polymerase (PARP) family of enzymes are central to the repair of single-strand DNA breaks. In 2018, the PARP inhibitor, olaparib, was approved by the FDA as monotherapy for the treatment of patients with germline *BRCA1*- or *BRCA2*-mutated metastatic breast cancer. This approval was supported by results from OlympiAD (NCT02000622) randomized controlled trial (Robson *et al.*, 2017), in which patients with *BRCA1* or *BRCA2*-associated metastatic breast cancer had a doubling of response rate (59.9% vs 28.8%) and progression-free survival (7.0 vs 4.2 months, $P < 0.001$) if they were randomized to olaparib alone compared with those who received standard chemotherapy. A subsequent randomized controlled trial in the potentially curable adjuvant and neoadjuvant setting, OlympiA (NCT02032823), has randomized 1800 breast cancer patients with germline *BRCA1* or *BRCA2* mutations to olaparib or placebo at the completion of their standard therapy. If positive, olaparib use will become more widespread in women for whom fertility is potentially an issue. Although other PARP inhibitors exist, none are at the stage of efficacy testing in early-stage breast cancer in a Phase III randomized trial.

Germline mutations in the *BRCA1* and *BRCA2* genes account for about 2.5% (or 42 000) of breast cancer cases diagnosed worldwide each year (Fidler *et al.*, 2017). In developed countries, most of these will be diagnosed at an early and potentially curable stage, commonly affecting women before they have started or completed childbearing. Consequently, there is an increasing need to assess the impacts of olaparib on quality of life, post-treatment. There is now considerable knowledge about many potential side-effects of olaparib from completed clinical trials (Pujade-Lauraine *et al.*, 2017; Robson *et al.*, 2017). However, understanding of the potential impacts on ovarian function or fertility is completely lacking, as this has been overlooked as an assessable endpoint in clinical trials.

Olaparib blocks PARP1 and 2, both of which are nuclear enzymes activated in response to DNA damage and which function to repair single-strand DNA breaks (Schreiber *et al.*, 2006). DNA breaks can accumulate in primordial follicle oocytes in response to chemotherapy (Winship *et al.*, 2018), or by endogenous DNA damage that occurs daily due to normal metabolic processes (Tubbs and Nussenzweig, 2017; Stringer *et al.*, 2018). By blocking single-strand break repair, PARP inhibition can generate double stand DNA breaks. Ordinarily, these may still be repaired by the homologous recombination DNA repair pathway, leading to cell survival. But when paired with a germline mutation of a key mediator of homologous recombination, such as *BRCA1* or *BRCA2*, and somatic loss or mutation of the other allele in the tumour cells during tumourigenesis, the BRCA1 and BRCA2 proteins are rendered non-functional, resulting in synthetic lethality. In the future, adjuvant breast cancer patients who receive olaparib will mostly have also received previous chemotherapy, including alkylating,

platinum, anthracycline and taxane agents. From studies in mice and women, each of these agents exerts different levels of toxicity to the ovary.

PARP1 and its downstream target, poly(ADP)-ribose (PAR), have previously been localized to the nuclei of oocytes throughout follicular development; from the primordial to the secondary follicle stages (Qian et al., 2010). Since oocytes produce PARP and PAR proteins and are known to be exquisitely sensitive to DNA damage (reviewed by Morgan et al. (2012), Bedoschi et al. (2016), Winship et al. (2018)), we hypothesized that even in a wild-type setting, olaparib may exacerbate chemotherapy-mediated ovarian damage and follicle loss. In this study, we aimed to comprehensively examine the impacts of olaparib alone, or following single agent chemotherapy, on the ovary in wild-type mice.

Materials and methods

Animals, treatments and tissue collection

Female 8-week-old (reproductively young; sexually mature) C57BL/6 mice were housed in a temperature-controlled high barrier facility (Monash University ARL), with free access to food and water, under a 12 h light-dark cycle. All animal procedures and experiments were performed in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals and approved by the Monash Animal Research Platform Animal Ethics Committee. Mice were weighed prior to intraperitoneal injection with either a single dose of 75 mg/kg per body weight cyclophosphamide (Sigma-Aldrich) (Goldman et al., 2017), 10 mg/kg doxorubicin (Sigma-Aldrich) (Kerr et al., 2012), 80 mg/kg carboplatin (Sigma-Aldrich) (Kano et al., 2017), 7.5 mg/kg paclitaxel (Sigma-Aldrich) (Gucer et al., 2001) or saline vehicle control on Day 0. These doses were chosen based on their ability to partially deplete primordial follicles in mice. From Days 1 to 28, mice received one daily subcutaneous injection with either 50 mg/kg olaparib (MedChemExpress, #HY-10162-1G) in 10% dimethylsulphoxide and saline containing 10% w/v 2-hydroxypropyl-β-cyclodextrin, or saline vehicle control containing 10% DMSO and 10% w/v 2-hydroxypropyl-β-cyclodextrin ($n = 5$/treatment group). This dose frequency and duration of administration was chosen based on preclinical data in mouse models of breast cancer, in which olaparib successfully reduced tumour burden (Rottenberg et al., 2008). The treatment regimen is also physiologically relevant to women. Women on the OlympiAD trial received 300 mg twice daily for up to 12 months, following chemotherapy (Robson et al., 2017). Based on an approximate 80-year human lifespan, this equates to approximately 28 days in mice. Two mice treated with carboplatin ($n = 1$ *olaparib;* $n = 1$ *saline*) were culled prior to the study endpoint due to rapid body weight loss. All other mice were healthy. On Day 15 of the study, oestrous cycling was monitored by vaginal cytology for 14 consecutive days using the Rapid Diff Stain Kit (Australian Biostain), as detailed previously (Byers et al., 2012). At 24 h following final treatment (Day 28 + 24 h), mice were humanely killed by isoflurane inhalation, followed by terminal cardiac puncture to collect peripheral blood. Serum was separated by centrifugation, then stored at −80°C. Mice were weighed at necropsy and ovaries harvested and fixed in 10% (vol/vol) neutral buffered formalin solution for 24h and paraffin embedded.

Follicle counts

To estimate ovarian follicle numbers, one paraffin embedded ovary per animal was exhaustively sectioned at 5 µm and every ninth tissue section was collected and stained with periodic acid-Schiff and haematoxylin. Whole tissue images were captured on the Aperio Digital Pathology Slide Scanner (Leica Biosystems) at ×20 objective, then visualized by a blinded assessor using Aperio ImageScope software (Leica Biosystems). Total number of primordial, transitional, primary follicles was quantified in every ninth section of each ovary and the total number of secondary and antral follicles were counted in every 36th section using a similar strategy as previously described (Tilly, 2003; Hutt et al., 2006). Follicles were counted if the oocyte nucleus was present. Total follicle numbers were obtained by multiplying the raw counts of oocytes sampled to correct for sections not counted. Corpora lutea number was determined by direct counting of every 36th section encompassing the entire ovary.

Anti-Müllerian hormone ELISA

Serum anti-Müllerian hormone (AMH) concentrations were determined in duplicate using the Mouse AMH ELISA (Ansh Labs) according to the manufacturer's instructions and absorbance measured using the ClarioStar microplate reader (BMG Labtech).

Immunohistochemistry

Five slides containing four ovarian tissue sections (5 µm) per ovary per animal were systematically selected then deparaffinized in histolene, before being rehydrated in a graded series of ethanol. Antigen retrieval was performed with sodium citrate (pH 6). Endogenous peroxidases were quenched, and non-specific binding of antibodies was blocked with 10% normal goat serum in 0.1 M Tris, 150 mM NaCl and 0.1% v/v Tween buffer (TNT). Three tissue sections per slide were incubated with primary antibody against rabbit FOXL2 (1:400; gift from Dr Dagmar Wilhelm, University of Melbourne) in TNT overnight at 4°C, while negative isotype controls of non-immunized rabbit IgG (Vector Laboratories) were included on the remaining section of each slide. Tissue sections were incubated with biotinylated goat antibody against rabbit IgG (1:500; Vector Laboratories), followed by avidin-biotin peroxidase complex (Vector Laboratories), then 3,3′-diaminobenzidine and sections were counterstained with haematoxylin. Whole tissue section images were captured as above. Only the central FOXL2-stained tissue section was analysed per slide; however, adjacent FOXL2-stained sections were referred to confirm the absence or presence of primordial follicle oocyte nuclei. Total primordial follicle remnant number was counted in five tissue sections per animal.

Immunofluorescence

For immunofluorescence staining, six ovarian tissue sections per animal were deparaffinized, rehydrated and blocking and antigen retrieval performed. Phospho-histone (γ)H2AX (Ser139) rabbit monoclonal (1:200; Cell Signaling Technology #9718) and c-kit (1:500; Novus biologicals) primary antibodies, or rabbit and goat IgG isotype controls (Vector Laboratories), were applied in TNT overnight at 4°C. Tissues were incubated with Alexa568-conjugated donkey secondary antibodies to goat or rabbit IgG (1:500; Molecular Probes). Sections were mounted with ProLong Diamond Anti-Fade Mountant containing DAPI

(Thermofisher). Slides were analysed by fluorescent confocal microscopy using a Nikon C1 Upright Confocal microscope. Positive γH2AX follicles were defined by staining of at least one positive punctate nuclear focus in the oocyte, when the ooplasm was visualized (c-kit positive), and expressed as a proportion (%) of total follicles. Analyses were assessed by a blinded reviewer.

TUNEL assay

The Apop Tag Peroxidase In Situ Apoptosis Detection Kit (Millipore) was used according to the manufacturer's instructions. Images were captured as above. Four sections per were analysed. Follicles were classified as positive if the oocyte and/or ≥10% granulosa cells were positive and these were expressed as a proportion (%) of total follicles.

Statistical analysis

Data are presented as mean ± SEM and statistical analysis was performed using GraphPad Prism Software. Normality was tested using a Shapiro–Wilk normality test. Normally distributed data were analysed by Student's *t*-test to compare two groups. Non-normally distributed data (growing follicle counts, corpora lutea counts and the proportions of positive-stained follicles) were analysed using a Mann–Whitney test. Differences were considered significant when $P < 0.05$.

Results

Olaparib directly mediates primordial follicle oocyte loss in wild-type mice

Olaparib alone did not exert any obvious toxicity to the animals and body weights remained unchanged compared to controls throughout the study (Supplementary Fig. S1). Ovarian follicles were classified morphologically (Fig. 1A–D). Olaparib alone significantly depleted primordial follicles by 36% versus the control ($P < 0.05$; Fig. 1E). Primordial follicle loss was independent of follicle activation since olaparib did not alter growing follicle populations (Fig. 1E and F). Olaparib did not affect serum AMH concentrations (Fig. 1G), corpora lutea number (Fig. 1H) or oestrous cycling (Fig. 1I) compared to the saline control. Olaparib led to significant accumulation of primordial follicle remnants, while these were rarely detected in control ovaries ($P < 0.05$; Fig. 2A and B). DNA damage (γH2AX-positive positive oocytes) was undetectable in controls, but evident in approximately 10% of surviving primordial follicles in olaparib treated ovaries (Fig. 2C and D), although this difference was not statistically significant.

Olaparib does not exacerbate chemotherapy-mediated primordial follicle oocyte loss in wild-type mice

Each chemotherapeutic agent significantly reduced the number of primordial follicles compared to saline ($P < 0.05$, $P < 0.01$; Supplementary Fig. S2). However, olaparib treatment for 28 days did not enhance chemotherapy-mediated primordial follicle depletion, when compared with each respective chemotherapy group that received saline for 28 days (Fig. 3A). Cyclophosphamide had little impact on primary (Fig. 3B), secondary (Fig. 3C) and antral growing follicle populations (Fig. 3D), while doxorubicin and carboplatin depleted these populations compared to controls. Olaparib exerted no exacerbated impact on growing follicle populations in combination with cyclophosphamide, doxorubicin or carboplatin, compared to corresponding chemotherapeutic with saline (Fig. 3B–D). However, when combined with paclitaxel, olaparib significantly enhanced primary follicle numbers ($P < 0.05$; Fig. 3B), but reduced secondary follicle numbers compared to paclitaxel combined with saline ($P < 0.05$; Fig. 3C), while antral follicle numbers remained unchanged (Fig. 3D). Serum AMH levels were unchanged in cyclophosphamide and paclitaxel treated animals that received either olaparib or saline, when compared with saline only treated controls (Fig. 4A). However, doxorubicin and carboplatin treated animals that received either olaparib or saline had significantly decreased circulating AMH concentrations versus saline only controls animals (Fig. 4A). We found a significant reduction in the number of corpora lutea in mice that received cyclophosphamide and olaparib treatment, versus cyclophosphamide and saline, but olaparib did not result in any other changes when combined with all other chemotherapy agents, versus the respective chemotherapy combined with saline (Fig. 4B). Mice from each treatment group progressed through all stages of the oestrous cycle (Fig. 4C, Supplementary Fig. S3). However, each chemotherapy treatment resulted in mice spending a greater proportion of total time spent in oestrous compared to all other phases, but this was not altered by olaparib treatment (Fig. 4C, Supplementary Fig. S3).

Olaparib does not alter growing follicle atresia in mice

TUNEL staining was consistently absent in primordial and primary follicles but predominantly localized to the granulosa cells of growing secondary and antral follicles (Fig. 5A and B). Olaparib did not alter proportions of secondary (Fig. 5C) or antral (Fig. 5D) follicle atresia compared with saline. Furthermore, olaparib did not enhance the proportions of chemotherapy-mediated secondary (Fig. 5C) or antral (Fig. 5D) follicle atresia when compared to each respective chemotherapeutic combined with saline.

Discussion

Following a comprehensive *in vivo* animal study, we report that a 28-day PARP inhibition regimen reduced primordial follicles in mice, but did not exacerbate chemotherapy-medicated primordial follicle loss. PARP blockade alone did not alter growing follicle numbers, suggesting that olaparib-mediated primordial follicle depletion is likely to be independent of follicle activation. Accordingly, olaparib treatment led to unchanged CL numbers, oestrous cycling and serum AMH concentrations compared to controls. In addition to uncovering potential clinical implications of olaparib exposure for female fertility, we sought to understand the mechanisms of follicle loss. Immunohistochemical analyses demonstrated that primordial follicle remnants, containing intact granulosa cells but no oocyte, were significantly increased in olaparib treated ovaries compared with controls, indicating that olaparib likely causes direct primordial oocyte death. In support, a proportion of surviving primordial oocytes in olaparib treated animals had sustained DNA damage, indicating that PARPs are likely required to repair DNA

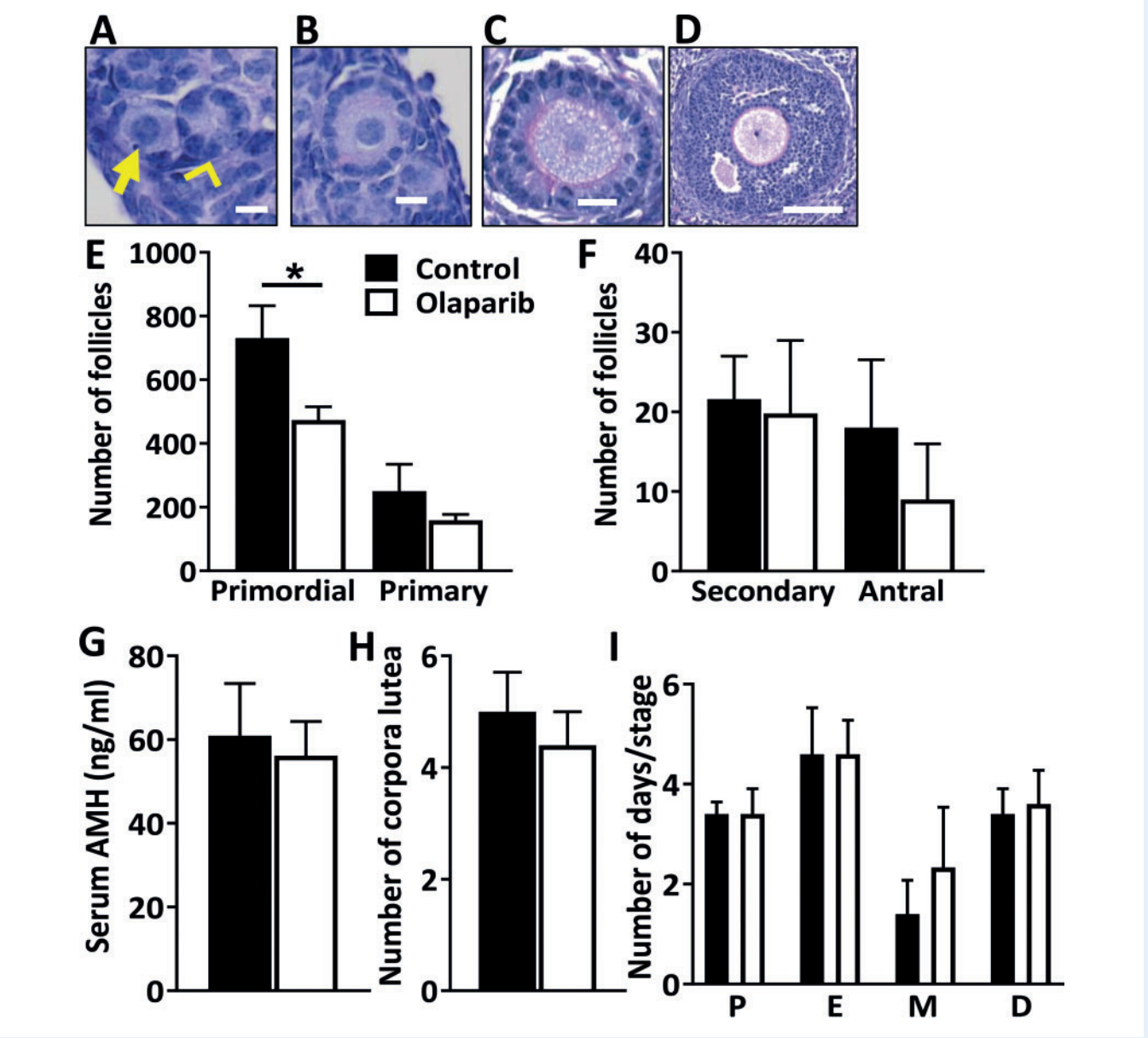

Figure 1. Olaparib depletes primordial follicle oocytes in mice. Representative photomicrographs of PAS stained (**A**) primordial follicle oocyte (solid arrow) and primordial follicle remnant (open arrow head); bar = 10 μm, (**B**) primary follicle; bar = 10 μm, (**C**) healthy secondary follicle; bar = 20 μm and (**D**) healthy antral follicle; bar = 100 μm. Female 8-week-old C57BL/6 mice received 50 mg/kg olaparib or vehicle control by daily subcutaneous injection from Days 1 to 28 and ovaries were harvested at Day 28 + 24 h. The number of healthy (**E**) primordial and primary and (**F**) secondary and antral follicles were quantified. (**G**) AMH concentrations were quantified in serum collected at Day 28 + 24 h following final treatment by ELISA. (**H**) Total number of corpora lutea were counted per ovary. (**I**) Oestrous cycling was monitored by vaginal cytology for a 14 day period from Days 15 to 28 of the study. The proportion of days spent in each stage of the cycle was quantified (P, pro-oestrus; E, oestrus; M, metoestrus; D, di-oestrus). Data are mean ± SEM; *t*-test (for normally distributed data) or Mann–Whitney test (for non-normally distributed data); $^*P < 0.05$; $n = 5$/group.

damage sustained by oocytes as a consequence of normal endogenous cellular processes (i.e. in the absence of significant exogenous insult). Together, our data show for the first time that blocking intrinsic PARP activity disrupts primordial follicle oocyte maintenance and survival.

Olaparib blocks PARP1 and 2, although PARP1 contributes to over 90% of poly(ADP-ribosyl)ation after DNA damage (Robson *et al.*, 2017). As well as its key functional role for DNA repair, nuclear protein poly(ADP-ribosyl)ation can lead to epigenetic alterations in chromatin structure and transcription (Beneke and Burkle, 2004). However, in the present study, alternative roles for PARP inhibition, other than DNA repair, in primordial follicles were not investigated.

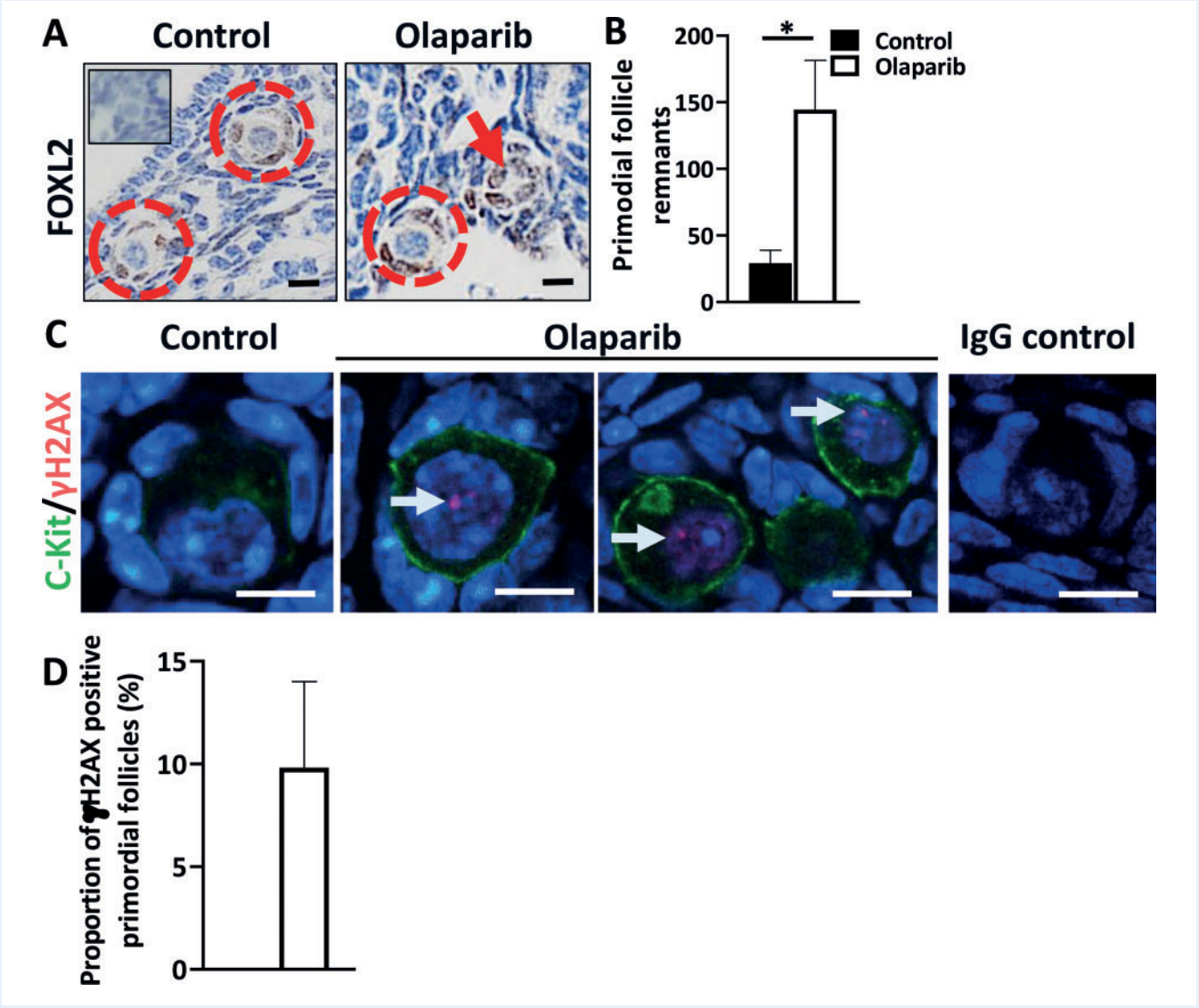

Figure 2. Olaparib treatment causes direct primordial follicle loss. (**A**) Representative photomicrographs of olaparib or vehicle control-treated mouse ovarian tissue sections stained by immunohistochemistry for FOXL2. Circles highlight intact primordial follicle oocytes; solid arrow highlights primordial follicle remnant characterized by granulosa cell rings (brown positive FOXL2 staining) and lacking an oocyte. Inset is negative control isotype IgG. (**B**) Quantification of the number of primordial follicle remnants. (**C**) Representative confocal images of olaparib or vehicle control-treated mouse ovarian tissue sections stained by immunofluorescence for γH2AX (red; DNA damage marker), c-kit (green; oocyte marker) or isotype IgG primary antibodies as a negative control and counterstained with DAPI (blue; nuclear marker). Solid arrow highlights red positive γH2AX foci. (**D**) Quantification of the proportion (%) of γH2AX positive stained follicles. Insets are negative controls. Bars = 10 μm. Data are mean ± SEM; *t*-test (for normally distributed data) or Mann–Whitney test (for non-normally distributed data); $^*P < 0.05$; $n = 5$/group.

PARP1 plays a critical role in the maintenance of chromosome stability at key stages of meiosis in the female germ line (Yang *et al.*, 2009). Interestingly, however, due to the redundant function of PARP1 with PARP2, PARP1-null female mice are fertile (Yang *et al.*, 2009), but the ovarian reserve of primordial follicles has not been quantified in these mice, nor has fertile lifespan been evaluated. This indicates that it is likely that the combination of both PARP1 and PARP2 blockade is fundamental to olaparib-mediated primordial follicle death, although this remains to be determined.

Interestingly, it has been reported that administration of 5-amninoisoquinolinone, an inhibitor of poly(ADP-ribosyl)ation, increased primordial follicle numbers and ovulated oocytes in 129S6/SvEv mice, without changes in secondary and antral follicles (Qian *et al.*, 2010). The discrepancy between this work and our findings following PARP1/2 inhibition with olaparib could be attributed to the many differences between each study, including the treatment regimen (9 vs 28 days), the follicle counting methods employed, the specificity and mode of action of the two inhibitors and/or timing of treatment (pubertal treatment versus adult). Importantly, this observation highlights the

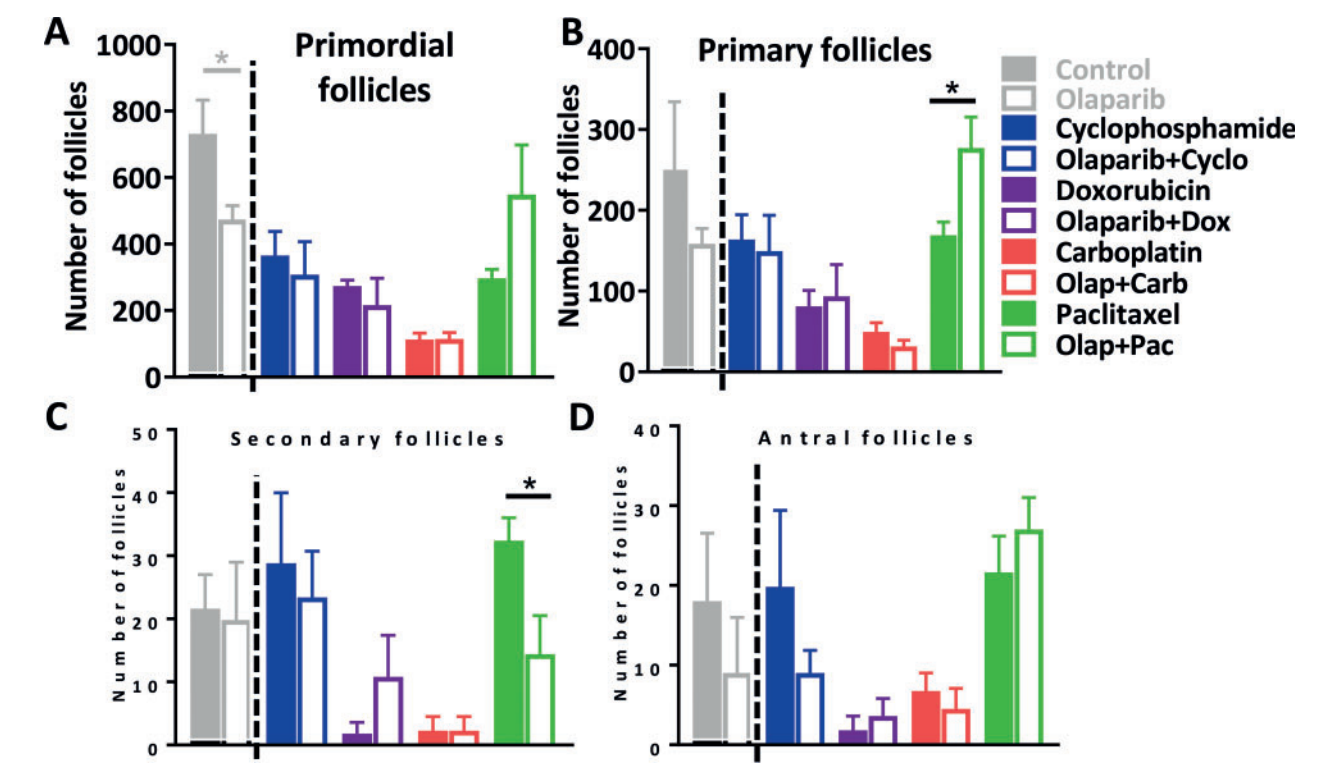

Figure 3. Olaparib does not exacerbate chemotherapy-mediated primordial follicle oocyte loss. On Day 0, female 8-week-old C57BL/6 mice were administered with a single intraperitoneal dose of either cyclophosphamide (75 mg/kg), doxorubicin (10 mg/kg), carboplatin (80 mg/kg), paclitaxel (7.5 mg/kg) or vehicle control. From Days 1 to 28, mice were administered subcutaneously with olaparib (50 mg/kg) or vehicle control and ovaries harvested at Day 28 + 24 h. Olaparib data from Fig. 1 are presented for clarity but separated by dotted lines. The number of healthy (**A**) primordial, (**B**) primary, (**C**) secondary and (**D**) antral follicles were quantified. Data are mean ± SEM; *t*-test between pairs (for normally distributed data) or Mann–Whitney test between pairs (for non-normally distributed data); $^*P < 0.05$; $n = 4$–5/group.

requirement to test the impacts of new PARP inhibitors on the female reproductive tract in preclinical studies, as they become available for clinical administration.

Serum AMH concentrations and menstrual cycling are used clinically as surrogate indicators of ovarian damage in response to cancer therapies (reviewed (Bedoschi *et al.*, 2016)). However, AMH is primarily secreted from granulosa cells of growing follicles, but not primordial follicles. Unfortunately, direct changes to the primordial follicle pool are impossible to quantify in women, due to a lack of available techniques. But, our data using a mouse model prove that olaparib depletes primordial follicles *in vivo*. On the contrary, olaparib did not affect growing follicles, ovulation, oestrous cycling nor serum AMH concentrations. This advocates that in a clinical setting, it is possible for olaparib-induced depletion of primordial follicles to go undetected, until premature ovarian insufficiency and early menopause ensue. Unfortunately, it may be many years before clinical data on fertility outcomes for women treated with PARP inhibitors becomes available.

Women treated with olaparib will receive multiple cycles of combination chemotherapy. However, for this study, we administered mice with a single dose of a single chemotherapy to ensure that we could systematically evaluate individual chemotherapy combinations with olaparib to provide insight into which drugs exert the most ovarian damage, and whether olaparib may enhance the level of chemotherapy-mediated damage. Surprisingly, however, we found no exacerbated effects of olaparib treatment on primordial follicle oocyte loss in mice treated with a single dose of four different chemotherapies. This is possibly attributed to the severe follicle depletion observed, even in response to a single dose of each chemotherapy, making it difficult to detect any additive effects of the combined treatment, and this is the subject of ongoing investigation.

Importantly, based on current data, women who receive olaparib will carry a germline *BRCA1* or *BRCA2* mutation, and based on our current findings in wild-type mice, it is hypothesized that the extent of olaparib-mediated follicle depletion may be enhanced in the context of impaired BRCA1 or BRCA2 function. Heterozygous loss of *BRCA1* in mice hinders reproductive capacity, due to diminished primordial follicle number and an age-related increase in DNA double-strand breaks in surviving oocytes, compared to wild-type mice (Titus *et al.*, 2013). Likewise, in women, serum AMH concentrations were reported to be significantly reduced in women with *BRCA1*, but not *BRCA2* mutations, compared to those without any mutation (Phillips *et al.*, 2016). This suggests that women carrying *BRCA1* mutations are predisposed to diminished oocyte reserves, supported by reports of premature menopause in these women (Rzepka-Gorska *et al.*, 2006; Lin *et al.*, 2017).

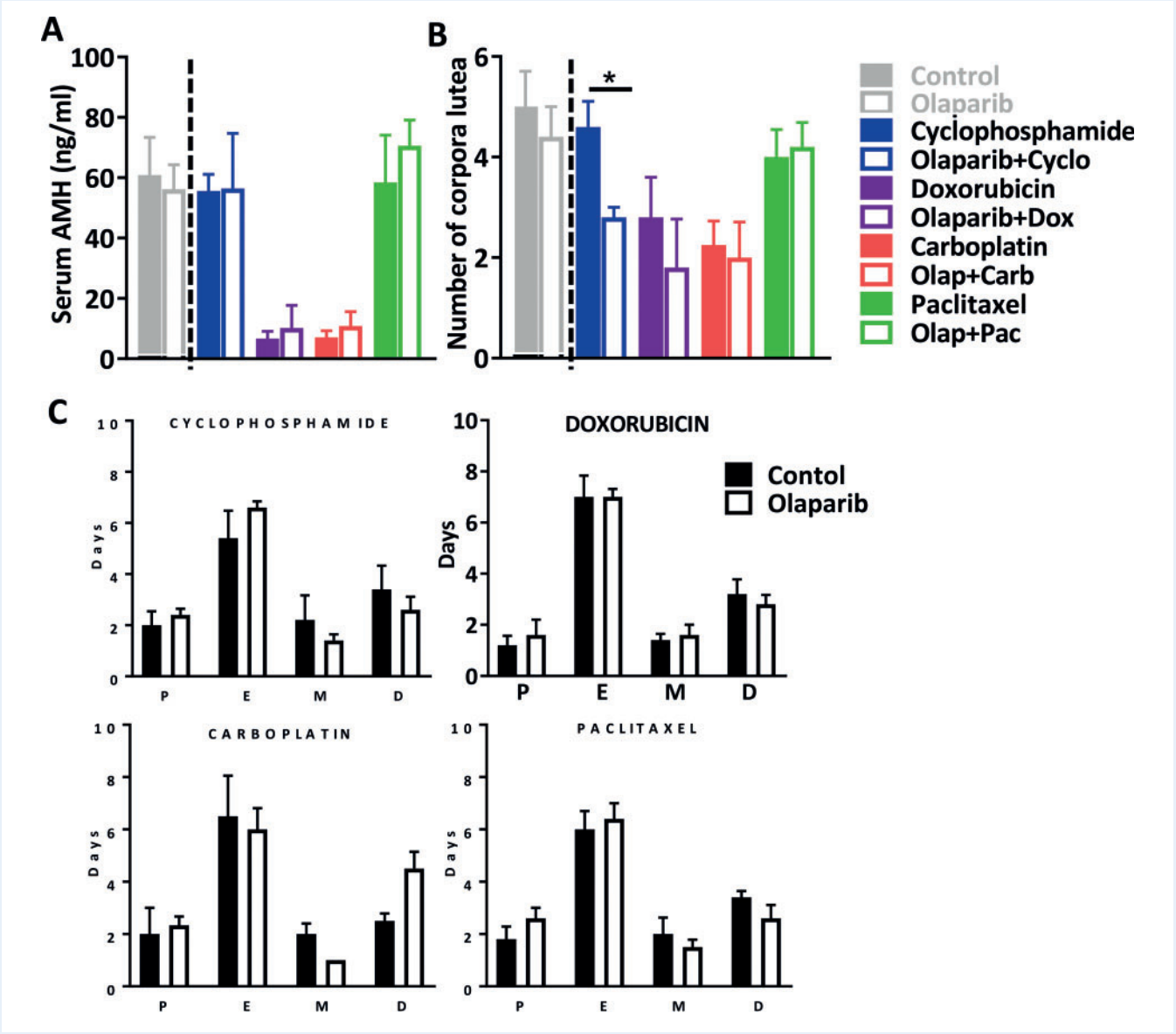

Figure 4. Olaparib does not alter serum AMH, oestrous cycling nor ovulation. On Day 0, female 8-week-old C57BL/6 mice were administered with a single intraperitoneal dose of either cyclophosphamide (75 mg/kg), doxorubicin (10 mg/kg), carboplatin (80 mg/kg), paclitaxel (7.5 mg/kg) or vehicle control. From Days 1 to 28, mice were administered subcutaneously with olaparib (50 mg/kg) or vehicle control and ovaries were harvested at Day 28 + 24 h. Olaparib data from Fig. 1 are presented for clarity but separated by dotted lines. (**A**) AMH concentrations were quantified in serum collected at Day 28 + 24 h following final treatment, by ELISA. (**B**) Total number of corpora lutea were counted per ovary. (**C**) Oestrous cycling was monitored by vaginal cytology for a 14 day period from Days 15 to 28 of the study. The proportion of days spent in each stage of the cycle was quantified (P, pro-oestrus; E, oestrus; M, metoestrus; D, di-oestrus). Data are mean ± SEM; *t*-test between pairs (for normally distributed data) or Mann–Whitney test between pairs (for non-normally distributed data); $^*P < 0.05$; $n = 4$–5/group.

The fact that fertility is likely already compromised in *BRCA1* mutation carriers highlights the importance of understanding the precise sensitivity of oocytes with *BRCA1* mutations to olaparib, and this should be the focus of future investigations.

Infertility is a major concern of young female cancer survivors (Letourneau *et al.*, 2012; Ruddy and Partridge, 2012). Many *BRCA1* and *BRCA2* mutation carriers are diagnosed with breast cancer in their 20s and 30s (Kuchenbaecker *et al.*, 2017), before they have completed childbearing. Furthermore, their risk of the other major BRCA-associated cancers (i.e. fallopian tube and ovarian cancer) is high enough to require risk-reducing bilateral salpingo-oophorectomy. Thus, fertility preservation is a concern for these women.

As cancer survival rates have increased over the past few decades, and the landscape of cancer therapies shifts towards more targeted therapies, carefully defining the impacts of new cancer therapies on fertility and the germline are becoming increasing important. Overall,

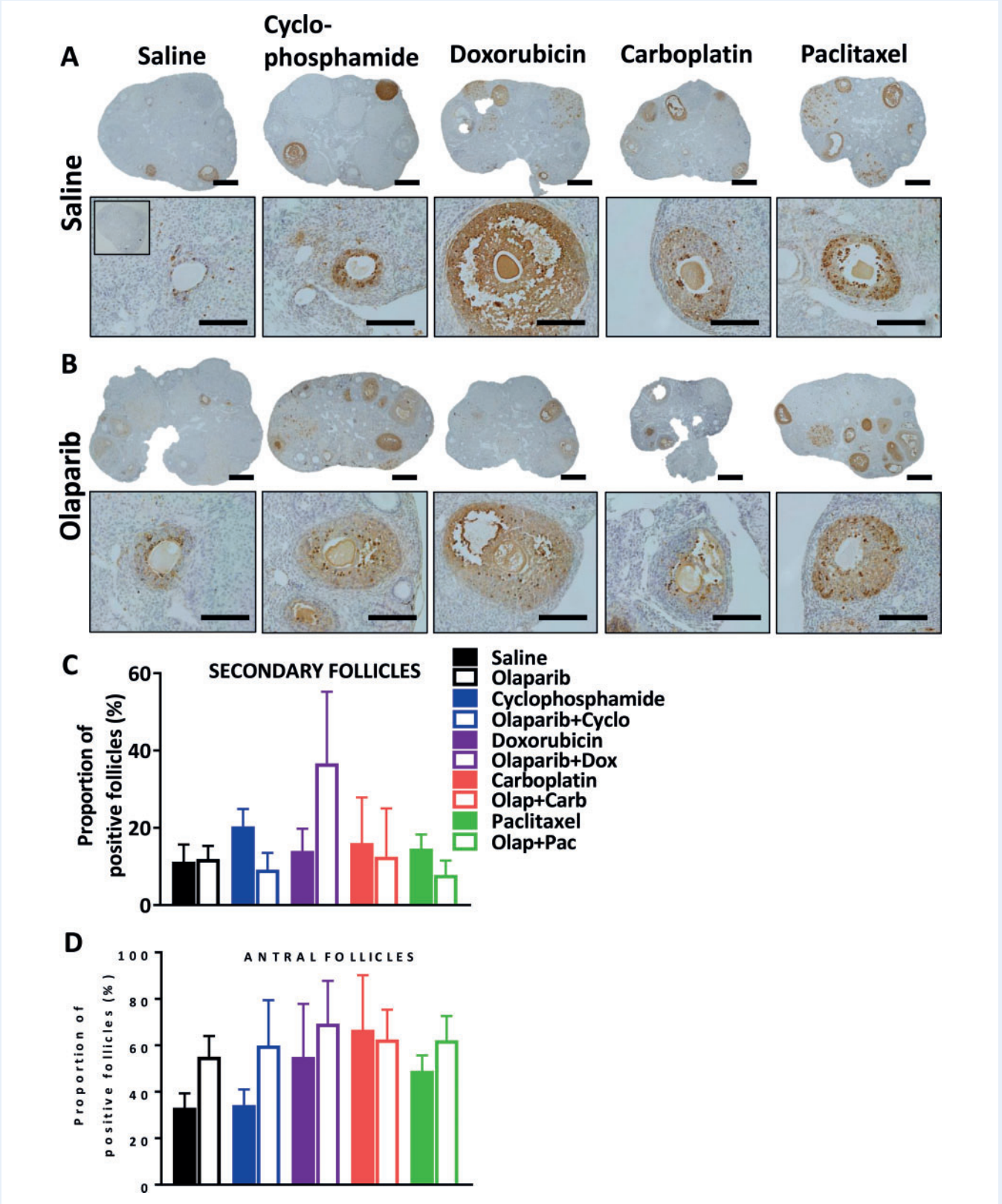

Figure 5. Olaparib does not alter growing follicle atresia. Analysis of follicle atresia in 8-week-old treated mouse ovaries at Day 28 + 24 h. Representative images of TUNEL-stained whole ovarian sections from mice treated with saline, cyclophosphamide, doxorubicin, carboplatin or paclitaxel on Day 0 and then either (**A**) saline or (**B**) olaparib from Days 1 to 28. Upper panels; scale bars 200 μm. Lower panels; scale bars 100 μm. Negative controls were included in each run. Quantification of the proportion (%) of TUNEL positive (**C**) secondary and (**D**) antral follicles. Data are mean ± SEM; Mann–Whitney test; $n = 4$–5/group.

our data emphasize the need for rigorous preclinical research to establish the potential of new and existing anti-cancer treatments to damage the ovary and impair fertility in women. Importantly, mice are a physiologically relevant model for such studies, although confirming our findings ovarian tissue biopsies and/or sera samples from women who have received olaparib should be the subject of further investigations.

Conclusions

Since direct measures of primordial follicle number are not possible in women, our data presented here have important clinical implications. Female cancer patients may present clinically with regular menstrual cycles and serum AMH concentrations within the normal range after olaparib treatment, but, unknowingly have a significantly depleted ovarian reserve of primordial follicles. Diminished ovarian reserve leads to infertility and premature menopause. Therefore, fertility preservation counselling should be considered for young female patients prior to olaparib treatment. Together, our findings provide the first evidence that under physiological circumstances, active DNA repair is essential for primordial follicle maintenance and survival. Where deficient, normalizing DNA repair capacity is a promising strategy for improved fertility.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

K.J.H. and K.A.P. conceived the study. K.J.H., A.L.W. and K.A.P. designed the study. A.L.W., M.G., C.L.R. and U.S. performed experiments. A.L.W., M.G., U.S. and K.J.H. analysed and interpreted the data. A.L.W. wrote the manuscript. All authors edited the manuscript.

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Conflict of interest

K.A.P. is the Breast Cancer Trials (Australia) Study Chair for the OlympiA clinical trial which is sponsored by AstraZeneca, the manufacturer of olaparib. All other authors declare no competing financial or other interests.

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