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ORIGINAL ARTICLE *Infertility*

Impact of first-line cancer treatment on the follicle quality in cryopreserved ovarian samples from girls and young women

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STUDY QUESTION: Does first-line chemotherapy affect the quality of ovarian pre-antral follicles and stromal tissue in a population of young patients?

SUMMARY ANSWER: Exposure to first-line chemotherapy significantly impacts follicle viability, size of residual intact follicles, steroid secretion in culture and quality of the stromal compartment.

WHAT IS KNOWN ALREADY: First-line chemotherapy is considered to have a low gonadotoxic potential, and as such, does not represent an indication for fertility preservation. Studies investigating the effects of chemotherapy on the quality of ovarian tissue stored for fertility preservation in young patients are limited and the results sometimes contradictory.

STUDY DESIGN, SIZE, DURATION: We conducted a retrospective cohort study including young patients referred to three centers (Helsinki, Oslo and Tampere) to perform ovarian tissue cryopreservation for fertility preservation between 2003 and 2018.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A total of 43 patients (age 1–24 years) were included in the study. A total of 25 were exposed to first-line chemotherapy before cryopreservation, whereas 18 patients were not. Density and size of follicles divided by developmental stages, prevalence of atretic follicles, health of the stromal compartment and functionality of the tissue in culture were evaluated and related to age and chemotherapy exposure. Activation of dormant follicles and DNA damage were also assessed.

MAIN RESULTS AND THE ROLE OF CHANCE: Patients exposed to first-line chemotherapy showed a significantly higher density of atretic primordial and intermediary follicles than untreated patients. The intact primordial and intermediary follicles were significantly smaller in size in patients exposed to chemotherapy. Production of steroids in culture was also significantly impaired and a higher content of collagen and DNA damage was observed in the stromal compartment of treated patients. Collectively, these observations may indicate reduced quality and developmental capacity of follicles as a consequence of first-line chemotherapy exposure. Neither increased activation of dormant follicles nor elevated levels of DNA damage in oocyte nuclei were found in patients exposed to chemotherapy.

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LIMITATIONS, REASONS FOR CAUTION: The two groups were not homogeneous in terms of age and the patients were exposed to different treatments, which did not allow us to distinguish the effect of specific agents. The limited material availability did not allow us to perform all the analyses on the entire set of patients.

WIDER IMPLICATION OF THE FINDINGS: This study provides for the first time a comprehensive analysis of the effects of first-line chemotherapy on the health, density and functionality of follicles categorized according to the developmental stage in patients under 24 years of age. When exposed to these treatments, patients were considered at low/medium risk of infertility. Our data suggest a profound impact of these relatively safe therapies on ovarian health and encourages further exploration of this effect in follow-up studies in order to optimize fertility preservation for young cancer patients.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by the Swedish Childhood Cancer Foundation, the Finnish Cancer Society, the Finnish Pediatric Research Foundation, the Väre Foundation for Pediatric Cancer Research, The Swedish Research Council, the Stockholm County Council (ALF project) and Karolinska Institutet. The authors have no conflict of interest to declare.

Key words: childhood cancer / chemotherapy / fertility preservation / follicle quality / DNA damage

Introduction

Premature ovarian insufficiency (POI) and infertility are common adverse effects of cancer treatment. Even when ovarian function is maintained, cancer survivors have a lower chance to achieve pregnancy than expected (Anderson *et al*., 2018; van Dorp *et al*., 2018). The toxic potential of chemo- and radiotherapy varies based on the agent's type and dose (Meirow and Nugent, 2001; Lee *et al*., 2006). Alkylating agents and radiation to the ovarian field are associated with the highest risk of infertility (Wallace *et al*., 2003; Donnez and Dolmans, 2014).

Cryopreservation of mature oocytes or embryos are established clinical procedures to preserve fertility in adult patients undergoing cancer treatments (Oktay *et al*., 2018). For women who cannot postpone the start of treatment, ovarian tissue cryopreservation (OTC) is the only remaining option. Post-pubertal, cryopreserved tissue retransplanted to the patient can restore ovarian function and fertility, with more than 130 live births reported worldwide (Donnez and Dolmans, 2017). OTC is the only feasible approach for fertility preservation in pre-pubertal girls, for whom retrieval of mature oocytes is not an option (Jadoul *et al*., 2010; Wallace *et al*., 2016; Donnez and Dolmans, 2017; Oktay *et al*., 2018). This approach is, however, still considered experimental. Ovarian tissue stored from pediatric patients has been successfully used for induction of puberty (Poirot *et al*., 2012; Ernst *et al*., 2013). Recently, two reports describe successful pregnancies stemming from transplantation of ovarian tissue collected when the patients were 13 (Demeestere *et al*., 2015) and 9 years old (Matthews *et al*., 2018).

The major centers routinely carrying out OTC offer the procedure when the estimated risk of developing POI is greater than 50%, based on the type of disease and treatment (Donnez *et al*., 2013; Wallace *et al*., 2016; Donnez and Dolmans, 2017; Jensen *et al*., 2017). At diagnosis, most girls are at low/medium risk of infertility and cryopreservation will not be offered. However, some patients will fall in the high-risk category later on due to poor treatment response or disease relapse and will hence be offered OTC after the initiation of treatments (Wallace *et al*., 2014).

A limited number of reports describe the effects of chemotherapy on ovarian follicles in pediatric populations (Marcello *et al*., 1990; Familiari *et al*., 1993; Abir *et al*., 2008; Fabbri *et al*., 2012; Asadi Azarbaijani *et al*., 2015; Duncan *et al*., 2015; El Issaoui *et al*., 2016). A normal density of viable pre-antral follicles has been observed in patients exposed to chemotherapy, compared to non-exposed ones, especially at younger ages (Abir *et al*., 2008; Fabbri *et al*., 2012; El Issaoui *et al*., 2016). However, Abir *et al*. (2008, 2016) reported a deterioration in follicular ultrastructure in patients exposed to chemotherapy before OTC. We have previously described decreased *in vitro* survival and maturation of ovarian follicles from cancer patients aged 1–35 years exposed to chemotherapy (Asadi Azarbaijani *et al*., 2015).

In the present study, we further characterized the effects of exposure to first-line chemotherapy on pre-antral follicles and ovarian stroma in a cohort of girls and young women with cancer.

Materials and Methods

Study population

A total of 43 young patients who underwent fertility preservation between 2003 and 2018 were included. The hospital, age, diagnosis, treatment and cumulative doses of alkylating agents and anthracyclines [cyclophosphamide equivalent doses (CEDs; Green *et al*., 2014) and isotoxic dose equivalents (DIE), respectively (Shankar *et al*., 2008) (Supplementary Materials and Methods)] are listed in Table I. Table I further indicates the use of samples in the different assays. As the material is very limited and precious, we could not carry out every analysis in all samples. Altogether, 27 of the samples in this study were from the same cohort used in our previous work (Asadi Azarbaijani *et al*., 2015).

Ethical approval

Ovarian tissue samples were donated by fertility preservation patients treated at the Children's Hospital of the University Central Hospital of Helsinki (Finland), the Department of Gynecology of the University Hospital of Tampere (Finland) and the Department of Gynecology of the Oslo University Hospital (Norway). Adult patients from Tampere and Oslo were offered OTC as a part of fertility preservation program, and they signed an informed consent for quality control of ovarian tissue including morphological analysis and *in vitro* culture. Requirements for further ethical licenses were discussed with Oslo University Hospital, Finnish National Supervisory Authority for Welfare and Health and Finnish Medicines Agency. Since all patients had given their written informed consent concerning the use of the material for scientific work, no further ethical permits were required in accordance with the Medial Research Act (488/1999) and Act on the Medical Use of Human Organs, Tissues and Cells (101/2001).

Table I Main clinical characteristics of the study population and type of assays performed for each patient.

Pt	Site	First-line treatment		TFL	Age	Dx	Assays					
		CED	DIE	days	years		Histology	Steroid	H2AX	FOXO-3A	Trichrome	Tunel
1	H	31560	300	7	12	ES	1	1	0	0	0	0
2	H	3600	210	11	5	ALL	1	1	0	0	1	0
3	H	2000	120	28	1	ALL	1	1	0	0	0	0
4	H	0	300	30	6	AML	1	1	0	0	1	1
5	H	0	320	44	4	AML	0	0	0	0	0	1
6	H	20840	225	55	1	NBL	1	1	0	0	1	0
7	H	11400	120	30	2	NBL	1	1	0	0	1	0
8	H	9496	260	9	15	ALL	1	1	0	0	0	0
9	H	6000	450	26	11	ALL	1	1	0	0	1	0
10	H	4100	180	18	15	NHL	1	1	0	0	0	0
11	H	2000	120	18	16	ALL	1	1	0	0	0	0
12	H	10248	90	17	5	RMS	1	1	1	0	0	0
13	O	8540	120	14	10	RMS	1	1	0	1	0	0
14	O	6200	300	35	20	NHL	1	1	1	1	1	1
15	O	4800	200	21	24	ALL	1	1	0	1	0	1
16	H	5589	135	55	3	NBL	0	0	1	0	0	0
17	H	23676	360	26	7	ES	0	0	1	0	0	0
18	H	7332	240	20	16	RMS	0	0	1	0	0	1
19	H	1272	0	8	2	PNET	0	0	1	0	0	0
20	H	5853	240	15	16	RMS	0	0	1	0	0	0
21	H	2100	0	48	4	NBL	0	0	1	0	0	0
22	H	5928	0	26	1	PNET	0	0	1	0	0	0
23	H	39593	360	30	11	RMS	0	0	1	0	0	0
24	H	4000	150	25	3	NBL	0	0	0	0	0	1
25	H	1900	60	11	10	WT	0	0	0	0	0	1
26	H	0	0	-	15	AA	1	1	1	0	0	1
27	O	0	0	-	12	ES	1	1	0	1	1	1
28	O	0	0	-	23	HL	1	1	0	0	1	0
29	O	0	0	-	20	NHL	1	1	0	0	1	0
30	O	0	0	-	16	OS	1	1	0	0	1	0
31	O	0	0	-	14	HL	1	1	1	1	1	0
32	O	0	0	-	24	ES	1	1	0	1	1	1
33	O	0	0	-	15	NBL	1	1	0	0	0	0
34	O	0	0	-	15	NHL	1	1	0	0	0	0
35	O	0	0	-	19	NHL	1	1	1	1	0	1
36	O	0	0	-	22	ALL	1	1	1	1	0	0
37	T	0	0	-	19	ALL	1	1	0	0	0	0
38	T	0	0	-	19	ALL	1	1	0	0	0	0
39	H	0	0	-	3	SCID	0	0	1	0	0	1
40	H	0	0	-	7	AA	0	0	1	0	0	0
41	H	0	0	-	8	CML	0	0	1	0	0	0
42	H	0	0	-	20	PNET	0	0	1	0	0	0
43	H	0	0	-	2	WT	0	0	0	0	0	1

AA, aplastic anemia; ALL, acute lymphoblastic leukemia; AML, acute mieloid leukemia; CED, cyclophosphamide equivalent doses (mg/m^2); CML, chronic myeloid leukemia; DIE, doxorubicin isotoxic equivalent (mg/m^2); Dx, diagnosis; ES, Ewing sarcoma; H, Helsinki; HL, Hodgkin lymphoma; NBL, neuroblastoma; NHL, non-Hodgkin lymphoma; O, Oslo; OS, osteosarcoma; PNET, primitive neuroectodermal tumour; Pt, patient; RMS, rhabdomyosarcoma; SCID, severe combined immunodeficiency; T, Tampere; TFL, time from last chemotherapy dose before ovarian tissue cryopreservation; WT, Wilms tumor. 0 = assay not performed; 1 = assay performed.

Pediatric patients were included from Children's Hospital in Helsinki where they participated in a fertility preservation research project approved by the Ethics Committee of Helsinki University Central Hospital (license number 340/13/03/03/2015). Written informed consents for pediatric patients were given by their guardians and by all age-appropriate patients..

Ovarian tissue handling

Ovarian cortical tissue was collected from a total of 43 patients (Table I). For 27 patients, tissue was fixed in formalin prior to cryopreservation and used for immunohistochemical processing. For 11 of these patients and 16 others (total 27 patients), frozen thawed ovarian tissue (ca $2 \times 3 \times 1.5$ mm^3) was fixed in Bouin's solution and used for morphometric evaluation as described earlier (Asadi Azarbaijani *et al*., 2015).

Morphometric analysis

Two blinded observers evaluated a subset of 10 sections independently and the results showed an inter-observer concordance above 90%. Following this, each observer evaluated half of the study material. Hematoxylin–eosin stained sections were digitalized using a spectral scanner (Vectra 3 system, Perkin Elmer, MA, USA) and every fifth section was evaluated using PannoramicViewer software (3DHistech, Budapest, Hungary). In order to avoid double counting, only follicles with a clear oocyte nucleus were counted and classified according to previously established morphological criteria (Gougeon, 1996): (i) primordial follicles, oocyte surrounded by a single layer of flattened granulosa cells; (ii) intermediary follicles, oocyte surrounded by a single layer of flattened and at least two cuboidal granulosa cells; (iii) primary follicles, oocyte surrounded by one complete layer of cuboidal granulosa cells; and (iv) secondary follicles, oocyte surrounded by more than one layer of cuboidal granulosa cells. Follicle health was assessed based on follicle shape, oocyte nucleus, oocyte cytoplasm and granulosa cells. In particular, a follicle was considered intact if the round shape was preserved, there was no contraction of the cytoplasm, the oocyte nucleus was centrally located, round and not pyknotic, if there was full contact between the oocyte and the surrounding granulosa cells and no eosinophilia of the cytoplasm. Atretic follicles were those with a distorted follicle shape, dislocated or detached granulosa cells, non-circular or pyknotic oocyte nucleus or eosinophilic cytoplasm (Supplementary Fig. S1).

Mean follicle diameter and mean oocyte nucleus diameter was recorded for each annotated follicle by taking two perpendicular measurements using the follicle basement membrane or oocyte nuclear membrane as a reference. The arithmetic mean of the two measurements was used for final calculations..

Staining of ovarian tissue sections

Fork head box O3A (FOXO3A) analysis was performed in one section 'per' patient in 8 patients (3 treated and 5 untreated) and gammaH2AX (H2A histone family member X phosphorylated on serine 139) analysis in one section 'per' patient in 18 patients (8 untreated and 10 treated). In brief, following antigen retrieval, slides were blocked in 5% BSA and normal donkey serum (S30, EMD Millipore, Darmstadt, Germany) and then incubated in polyclonal rabbit FOXO3A antibody (PA5-27145, Thermo Fisher Scientific, MA, USA), polyclonal rabbit gammaH2AX antibody (ab2893, Abcam, Cambridge, UK) or polyclonal rabbit IgG antibody (ABIN376827, antibodies-online, Aachen, Germany) as negative control overnight at 4°C. Primary antibodies were detected using donkey anti-rabbit Alexa Fluor 555 conjugated antibody (A31572, LifeTech, MA, USA) for 1 h at room temperature. Sections were mounted using VECTASHIELD mounting media with DAPI (H-1200, VectorLabs, CA, USA).

For detection of fibrotic areas in the ovarian stroma, trichrome staining was performed on samples fixed in Bouin's after thawing using trichrome stain kit (ab150686, Abcam, Cambridge, UK) and one section 'per' patient in 12 patients (6 untreated and 6 treated patients). Briefly, tissue sections were incubated in Weigert's iron hematoxylin for 5 min, followed by Biebrich scarlet/acid fuchsin solution for 15 min, phosphomolybdic/phosphotungstic acid solution for 10 min, aniline blue solution for 5 min and acetic acid solution for 3 min before dehydration (95% ethanol and 100% ethanol) and clearing (xylene for 10 min). Tissue sections were mounted using Pertex® (Histolab, Askim, Sweden).

For stromal cell death detection, TUNEL staining assay was carried out (11684795910, Roche, Penzberg, Germany) using freshly formalin-fixed paraffin-embedded samples and one section 'per' patient in 13 patients (6 untreated and 7 treated). In brief, de-paraffinized and rehydrated tissue sections were treated with proteinase K and incubated with TUNEL reaction mixture for 1 h at 37°C. Positive controls were treated with DNase I for 20 min at room temperature prior to TUNEL reaction. Negative controls were incubated without detection enzyme. Samples were mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector laboratories, Peterborought, UK).

Details of imaging of stained ovarian tissue sections are given in Supplementary Materials and Methods.

Steroid production in culture

Follicle function was evaluated by measuring steroid hormones in used culture media from ovarian tissue pieces thawed and cultured for 7 days as described earlier (Asadi Azarbaijani *et al*., 2015). Concentrations of cortisol, corticosterone, estradiol, androstenedione, 17a-OH-progesterone, dehydroepiandrosterone, dihydrotestosterone, testosterone and progesterone were quantified in media samples from 27 patients on Days 4 and 7 of culture, and in nine blank media samples as controls, using an established ultra-performance liquid chromatography tandem mass spectrometry method (Hao *et al*., 2018).

Statistical analyses

Statistical analyses were performed using the SigmaStat (v 11.00) package (SPSS, Inc., IL, USA), R (R Core Team, 2018) and RStudio program (RStudio Team, 2015) with the packages ggplot2 (Wickham, 2009), gplots (Warnes *et al*., 2016), reshape2 (Wickham, 2007) and dichromat (Lumley, 2013). Mann–Whitney *U* test was used to compare follicle densities, follicle and oocyte nucleus diameters, steroid concentrations, fibrotic areas, TUNEL signal and gammaH2AX foci numbers between the treated and the untreated patient group. Results are shown as medians and interquartile range. Outliers are defined as values that fall more than 1.5 times the interquartile range above the third quartile or below the first quartile and shown as dots in the figures. All values were included in statistical analyses. Chi-Square analysis was used to compare the proportions of different follicle classes in treated and untreated patients. Morphometric analysis data

were used to verify correlation between the morphological parameters and age, CED, DIE and steroid concentrations using Spearman's rank correlation. Forward stepwise regression was used for the evaluation of the major predictor when more than one variable correlated with morphological parameters in Spearman's analysis. Differences were considered statistically significant at $P < 0.05$.

Results

First-line chemotherapy affects follicle density and size

Follicular density was evaluated in ovarian samples from 14 treated and 13 untreated cancer patients (Fig. 1). A total of 3154 follicles (2408 in the treated and 746 in the untreated group) were counted. Representative histological images of ovarian tissue samples from both patient groups are shown in Fig. 1. The main clinical data are reported in Table I. The treated patients had received a median cumulative dose of 6100 CED (0–20,840 mg/m^2) and 205 DIE (90–450 mg/m^2) and were significantly younger than the patients who were not treated (Table II). The density of atretic primordial and intermediary follicles was higher in treated patients compared to untreated ones (Table II; Fig. 2A). Treated patients also had a significantly lower density of intact primary follicles compared to untreated patients. When the proportions of follicles were compared instead of densities, treated patients were found to have a significantly higher proportion of atretic follicles in all categories (Fig. 2B). The total follicle density did not differ between the treated and the untreated group (Table II).

In treated patients, intact and atretic primordial follicle and oocyte nucleus diameters were significantly smaller compared to those of untreated patients (Fig. 3; Table III). Intact intermediary follicles had also a significantly lower follicular diameter in treated patients.

CED was positively correlated with the total density of atretic follicles (i.e. the sum of atretic primordial, intermediary and primary follicles), atretic primordial follicles and atretic intermediary follicles and was negatively correlated with the density of total and intact primary follicles and with the diameter of intact intermediary follicles. DIE was positively correlated with the density of atretic total follicles, atretic primordial and atretic intermediary follicles and was negatively correlated with the density of intact primary follicles. DIE showed also a negative correlation with the follicular and nuclear diameter of intact primordial follicles and with the diameter of intact intermediary follicles. Age was inversely correlated with the density of atretic primordial follicles (Supplementary Table S1). Forward stepwise regression analysis showed that among age, CED and DIE, age was the major determinant of atretic total follicle density ($P = 0.017$), atretic primordial follicle density ($P = 0.026$), atretic intermediary follicle density ($P = 0.010$) and of the ratio between growing and total follicles ($P = 0.024$). The diameter of intact intermediary follicles and the density of intact primary follicles were instead mainly influenced by DIE ($P = 0.024$ and $P = 0.023$, respectively).

No increase in follicle activation in chemotherapy exposed ovarian tissue

As a measure of follicle recruitment, i.e. activation of follicle growth, the ratio of intact growing follicles to the total number of follicles was compared between treated and untreated patients. The ratio was significantly reduced in treated patients compared to untreated ones (Fig. 4A), suggesting that there is no increase in the recruitment of primordial follicles but rather a decrease in the number of growing follicles, consistent with the lower density of intact primary follicles observed in the treated group (Fig. 2A; Table II). We further studied follicle activation through FOXO3A immunostaining. Upon follicle activation, transcription factor FOXO3A is relocated from the nucleus

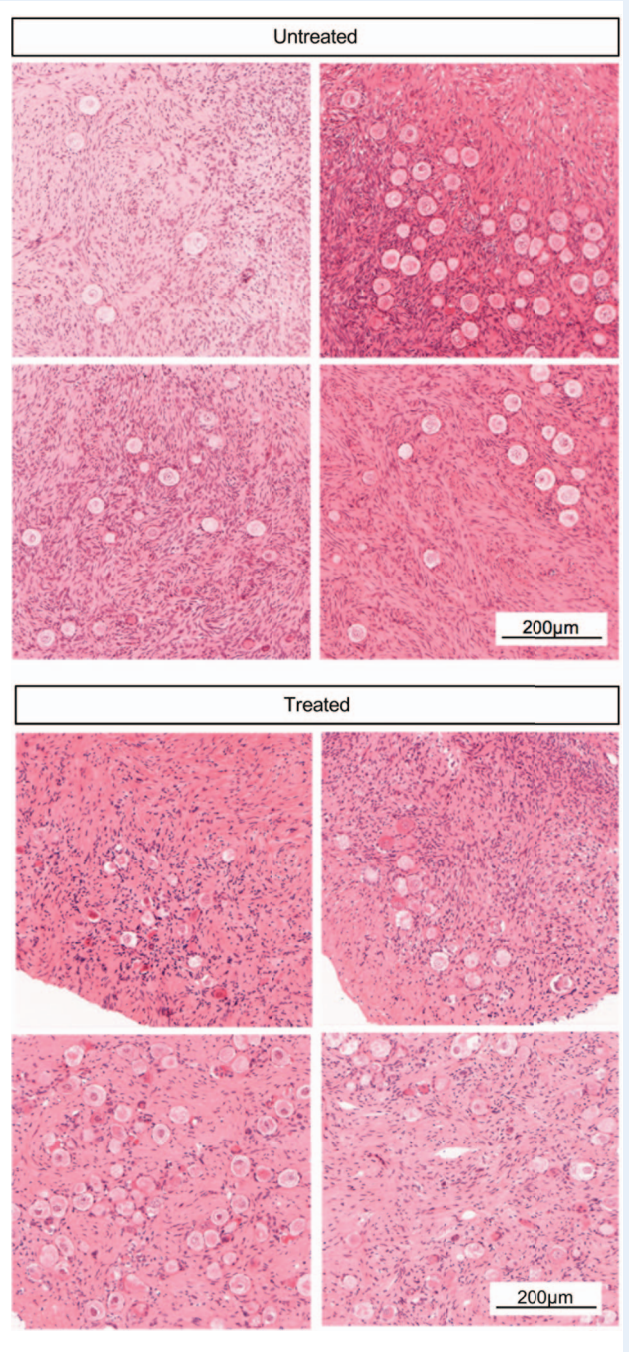

Figure 1 Representative images of ovarian tissue sections. Hematoxylin–eosin stained ovarian tissue sections from four patients treated with chemotherapy prior to OTC (Patient number 4, 2, 7, 6; clockwise) and four untreated patients (Patient number 31, 34, 28, 29; clockwise).

Table II Density of intact and atretic follicles (follicles/mm³) in cryopreserved human ovarian tissues from cancer patients untreated and treated with chemotherapy before ovarian tissue cryopreservation.

	Untreated (n=13)	Treated (n=14)	P
Age (y) mean± SD	17.9 ± 3.7	10.2 ± 7.2	**0.007**
CED (mg/m²)	0.0 (0.0)	6100(3200-10536)	**<0.001**
DIE (mg/m²)	0.0 (0.0)	205 (120-300)	**<0.001**
Total follicle density	421 (328-708)	764 (168-1,658)	0.452
Intact follicles	10.8 (0.0-231)	31.4 (0.0-154)	0.243
Atretic follicles	76.1 (34.4-332)	506 (81.6-1,488)	**0.027**
Primordial follicle density	142 (72.1-268)	225 (37.6-850)	0.593
Intact primordial follicles	105 (52.3-259)	73.0 (19.4-315)	0.511
Atretic primordial follicles	20.7 (0.0-34.5)	116 (13.4-621)	0.054
Intermediary follicle density	181 (156-462)	410 (137-736)	0.452
Intact intermediary follicles	136 (74.8-271)	82.9 (0.0-169)	0.138
Atretic intermediary follicles	49.2 (9.4-183)	308 (41.9-711)	**0.041**
Primary follicle density	22.6 (19.5-43.4)	3.3 (0.0-24.3)	**0.028**
Intact Primary follicles	20.6 (0.0-30.4)	0.0 (0.0)	**0.005**
Atretic Primary follicles	0.0 (0.0-21.2)	0.0 (0.0-22.3)	0.935

Data are expressed as median (quartiles), except for age that is expressed as mean (standard deviations). Comparison between untreated and treated subjects are reported. Bold characters emphasize significant P-values.

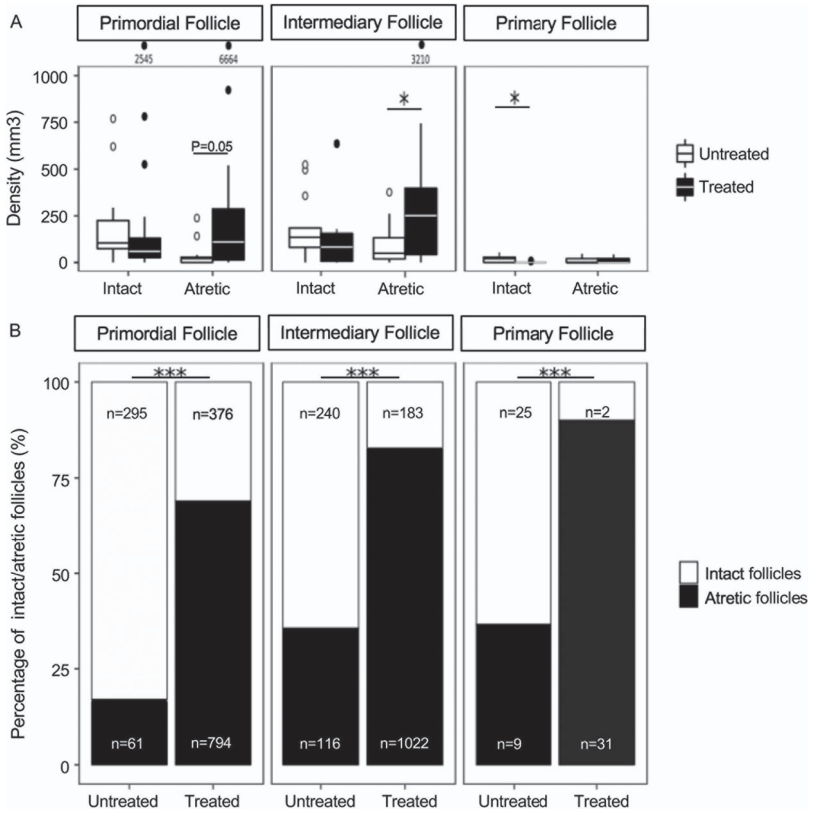

Figure 2 Follicle density is impacted by first-line chemotherapy. (**A**) Follicle densities of intact and atretic follicles at different stages of development (primordial, intermediary and primary). Atretic intermediary follicles were found to be increased in treated patients, while intact primary follicle density was decreased *($P < 0.05$). (**B**) Percentage of intact/atretic follicles of 14 treated and 13 untreated patients. A significantly higher percentage of atretic follicles of all developmental stages was observed in treated patients ***($P < 0.001$).

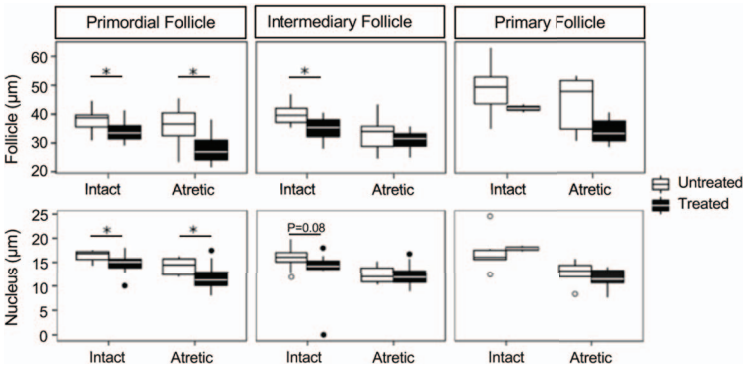

Figure 3 Size of follicle and oocyte nucleus is decreased by first-line chemotherapy. Box plots representing diameters of different stages of follicles and their oocyte nuclei of treated ($n = 14$; black bars) and untreated ($n = 13$; white bars) patients. Intact and atretic primordial follicles and their oocyte nuclei as well as intact intermediary follicles were significantly smaller in treated patients * ($P < 0.05$), while no difference in primary follicle and oocyte nucleus diameters was observed.

Table III Follicle and oocyte nucleus diameters (μm) of intact and atretic follicles in cryopreserved human ovarian tissues from cancer patients untreated and treated with chemotherapy before ovarian tissue cryopreservation (OTC).

	Untreated (n=13)	Treated (n=14)	P
Intact primordial follicles			
Follicle diameter	38.7 (35.1-40.0)	33.4 (31.2-36.4)	**0.042**
Nuclear diameter	16.8 (14.8-17.3)	15.1 (13.8-15.8)	**0.042**
Atretic primordial follicles			
Follicle diameter	36.6 (31.3-40.6)	26.9 (23.9-31.5)	**0.017**
Nuclear diameter	14.4 (12.3-15.9)	11.2 (9.6-13.1)	**0.043**
Intact intermediary follicles			
Follicle diameter	39.6 (36.8-43.1)	35.3 (32.1-38.7)	**0.009**
Nuclear diameter	15.9 (14.8-17.1)	14.1 (13.3-15.3)	0.081
Atretic intermediary follicles			
Follicle diameter	34.0 (27.5-36.7)	31.5 (27.1-33.2)	0.336
Nuclear diameter	11.9 (10.6-14.1)	11.8 (9.9-14.0)	0.780
Intact primary follicles			
Follicle diameter	49.3 (41.0-53.5)	42.0 (40.6-43.4)	0.333
Nuclear diameter	16.0 (15.5-17.8)	17.9 (17.3-18.5)	0.500
Atretic primary follicles			
Follicle diameter	47.8 (32.7-52.4)	33.3 (30.1-38.8)	0.177
Nuclear diameter	13.1 (10.3-15.0)	11.6 (10.0-13.7)	0.329

Data are expressed as median (quartiles). Comparison between untreated and treated subjects are reported. Bold characters emphasize significant P-values.

to the cytoplasm (Fig. 4B). All intact follicles of good morphology (judged by DAPI staining) had a clear nuclear localization of FOXO3A, suggestive of a dormant stage.

No increase in gammaH2AX expression in ovarian tissues exposed to chemotherapy

We evaluated DNA damage in our samples by gammaH2AX immunostaining. Only intact follicles were included in the analysis, as judged by DAPI stained follicle morphology. Our staining showed no difference in the number of gammaH2AX foci in oocyte nuclei from treated compared to untreated patients, suggesting no differences in DNA double-strand breaks between the groups ($P = 0.416$; Supplementary Fig. S2).

Ovarian tissue exposed to first-line chemotherapy produces less steroid hormones during culture

For evaluation of follicle function, we quantified production of steroid hormones during culture. Analysis of blank medium samples showed no steroids present in basal medium. Cortisol, corticosterone and dihydrotestosterone were undetectable in all samples. The average

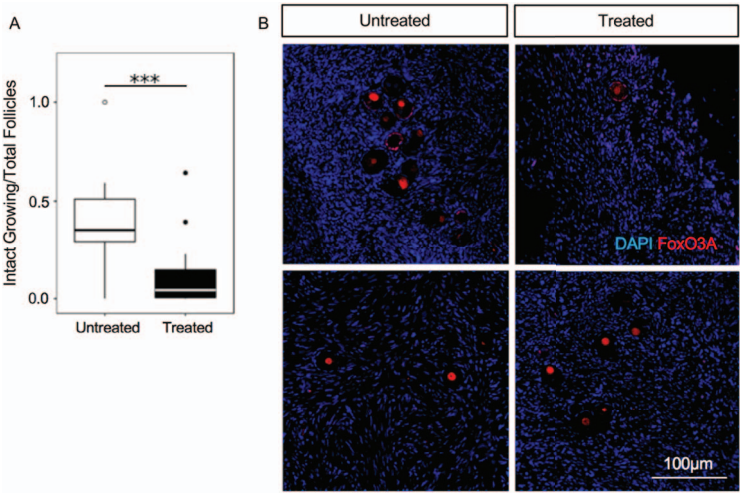

Figure 4 Exposure to chemotherapy before ovarian tissue cryopreservation does not increase activation of ovarian follicles. (**A**) Box plots showing a significantly smaller proportion of growing follicles in ovarian tissue of treated ($n = 14$) and untreated ($n = 13$) patients. For each patient, the number of intact growing (intermediary and primary) follicles was divided by the total number of intact and atretic follicles (primordial, intermediary and primary) *** ($P < 0.001$). (**B**) Immunofluorescence of ovarian tissue from a subset of treated ($n = 3$) and untreated ($n = 5$) patients labeled with fork head box O3A (FOXO3A) antibody shows no differences between the two groups. FOXO3A is located in the nuclei of oocytes and granulosa cells of both treated and untreated patients indicating non-activated follicles. Representative images of ovarian tissue sections are shown.

Table IV Steroid hormone concentrations in culture media of ovarian cortical strips from cancer patients untreated and treated with chemotherapy before ovarian tissue cryopreservation (OTC) at Day 4 and at Day 7 of culture.

Hormone (nM)	Day 4			Day 7		
	Untreated (n=13)	Treated (n=14)	P-value	Untreated (n=13)	Treated (n=14)	P-value
P4	13.0 (2.1-25.6)	0.8 (0.2-4.3)	**0.035**	24.6 (5.5-123.3)	3.7 (0.8-16.0)	0.073
17alphaOHP	0.1 (0.08-0.2)	0.04 (0.0-0.2)	0.3	0.3 (0.1-1.1)	0.2 (0.0-0.7)	0.27
A	0.6 (0.3-1.6)	0.2 (0.09-0.3)	**0.021**	1.6 (1.3-3.1)	0.5 (0.2-1.2)	**0.016**
T	0.1 (0.03-0.7)	0.01 (0.004-0.04)	**0.002**	0.6 (0.1-1.8)	0.05 (0.02-0.1)	**0.013**
E2	0.2 (0.0-1.5)	0.0 (0.0-0.1)	0.058	2.5 (0.2-8.5)	0.1 (0.0-0.5)	**0.048**

P4, progesterone; 17alphaOHP, 17 alpha hydroxyprogesterone; A, androstenedione; T, testosterone; E2, estradiol. P-value refers to the comparison of hormonal concentration in culture media between untreated and treated ovarian cortical strips. Bold characters emphasize significant P-values.

concentrations of estradiol, androstenedione, 17a-OH-progesterone, dehydroepiandrosterone, testosterone and progesterone increased over time in culture in both groups of patients, but were generally lower in treated compared to untreated patients (Table IV). In treated patients compared to untreated ones, androstenedione and testosterone concentrations were significantly lower on Days 4 and 7, progesterone was significantly lower on Day 4 and estradiol was significantly lower on Day 7 (Table IV). Steroid concentrations were positively correlated with density of intact and growing follicles at both time points (Supplementary Table SII).

First-line chemotherapy affects ovarian stromal tissue

As a measure of fibrosis in ovarian stroma, we carried out trichrome assay that detects newly formed collagen-rich (fibrotic) connective tissue in blue (Fig. 5A). The stroma of patients exposed to chemotherapy showed significantly larger areas of collagen with a greater intensity compared to untreated patients (Fig. 5B). DNA fragmentation was evaluated by TUNEL staining of ovarian tissue sections. All untreated patients showed low levels of TUNEL staining in the ovarian stroma, while treated patients showed TUNEL positive tissue areas to various degrees (Fig. 6A). Of the seven analyzed treated patients, five showed clear increase in TUNEL signal while two remained similar to untreated patients. When all patients were included in statistical analysis, TUNEL signal showed only borderline increase in treated compared to untreated patients ($P = 0.07$, Fig. 6B). When the two TUNEL-negative treated patient were removed from the analyses, the difference became significant ($P = 0.004$).

Discussion

First-line chemotherapy is regarded as low risk in terms of gonadotoxicity, and as such, does not represent an indication to perform

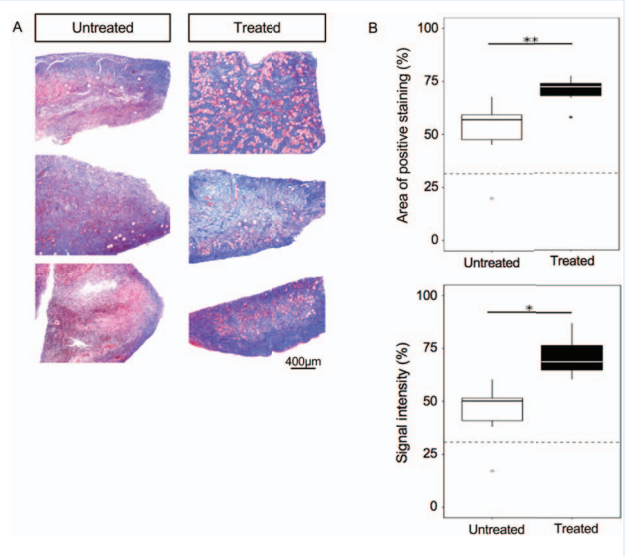

Figure 5 Exposure to chemotherapy before ovarian tissue cryopreservation increases collagen-rich stromal tissue areas. (**A**) Representative images of trichrome assay performed on ovarian tissue sections of treated ($n = 6$) and untreated patients ($n = 6$). Newly formed collagen fibers stain in blue. (**B**) Quantification of the blue component shows that the area of positive staining and the signal intensity are increased in ovarian stromal tissue of patients treated with chemotherapy before OTC. Dashed line indicates reference value of control adult ovarian tissue ($n = 1$) *($P < 0.05$), **($P < 0.005$).

OTC. This study provides, for the first time, a comprehensive analysis of the effects of first-line chemotherapy on pre-antral follicles and on the stromal compartment in girls and young women 24 years of age and below. We found a higher number of atretic follicles in ovaries exposed to first-line chemotherapy before OTC compared to those who were not. In addition, we found that treated patients had significantly smaller intact follicles in the earliest stages of development (primordial and intermediary). The diameters of untreated follicles and oocyte nuclei were in line with the previously reported normative values (Westergaard *et al*., 2007), whereas the diameters in the treated group were substantially smaller. This might suggest that even follicles that escape atresia after chemotherapy exposure exhibit significant morphological alterations that could have functional consequences. In addition to altered morphology, the density of intact primary follicles was significantly reduced in the treated group, possibly indicating a lower *in vivo* developmental potential of the follicles. These data support our previous *in vitro* culture study, where we reported an impairment of the developmental potential of ovarian follicles in samples collected after chemotherapy (Asadi Azarbaijani *et al*., 2015).

We observed that both the cumulative doses of anthracyclines and alkylating agents as well as the patient's age influence the size of intact intermediary follicles and their ability to progress toward the primary stage, with anthracyclines showing an independent effect. In clinical settings, anthracyclines are classified as safe in terms of risk of ovarian insufficiency. McLaughlin *et al*. (2017) reported that non-growing follicle density was not depleted but even increased in adult women after exposure to anthracyclines as part of the adriamycin, bleomycin, vinblastine and dacarbazine protocol for Hodgkin lymphoma. However, our observation of an adverse effect of anthracyclines in tissues cryopreserved for fertility preservation highlights that other first-line chemotherapeutics than those associated with long-term ovarian toxicity may also have an impact on follicle quality, at least in young patients.

Follicle activation

The 'burn-out' mechanism has been proposed as a potential mechanism of chemotherapy-induced infertility in mice (Kalich-Philosoph *et al*., 2013; Roness *et al*., 2016), bovine (Gavish *et al*., 2015) and human ovary (Lande *et al*., 2017). Nuclear localization of FOXO3A marks the dormant state of follicles (Li *et al*., 2010). All intact primordial follicles in our samples expressed FOXO3A in the nucleus. In addition, we observed a reduced ratio of growing to total follicles in treated *versus* untreated patients, leading us to reject the hypothesis of an increased activation of follicles in treated patients. It should be noted that the time since last treatment varied from 7 to 55 days, which could reduce chances of detecting possible temporary follicle activation.

DNA damage in follicles

We observed a similar amount of gammaH2AX foci in oocyte nuclei between treated and untreated patients. GammaH2AX is one of the first molecules recruited at DNA damage sites during the process of DNA repair. Mouse studies have shown that phosphorylation of H2AX happens in the oocyte nucleus around 10 min after DNA damage (Rogakou *et al*., 1998; Ji *et al*., 2017), reaching a peak after 24 h (Gonfloni *et al*., 2009; Petrillo *et al*., 2011; Rossi *et al*., 2017). Assuming that H2AX follows the same kinetics in human ovaries, we might have failed to detect higher levels, as chemotherapy has been administered 8–55 days before OTC in analyzed treated cases. Alternatively, gammaH2AX might not be a suitable marker for cell death after chemotherapy, which could instead be mediated by oxidative stress or indirectly by stromal damage (Tsai-Turton *et al*., 2007).

Follicle function

Analysis of media from ovarian cultures showed production of both theca and granulosa cell-specific hormones, with increasing concentrations over time in culture and levels comparable to those produced by cultured ovaries of adult patients (Hao *et al*., 2018). According to literature, theca and granulosa cells express steroidogenic enzymes from the secondary follicle stage (Andersen and Ezcurra, 2014). In our samples, the number of secondary follicles was very low and steroid production was positively associated with the density of intact intermediary and primary follicles. Very little is known about the steroidogenic activity of pre-pubertal ovaries. However, fetal ovaries are capable of producing estradiol already from the second trimester, i.e. when primordial follicle formation occurs and pre-granulosa cells appear (Fowler *et al*., 2011), suggesting that the steroids detected in our samples could be secreted by pre-antral follicles. Alternatively, somatic cells might acquire steroidogenic potential after exposure to the *in vitro* environment, as previously suggested (Oktem and Oktay, 2007). If this is the case, the overall lower steroid concentration in ovarian tissue from treated patients could

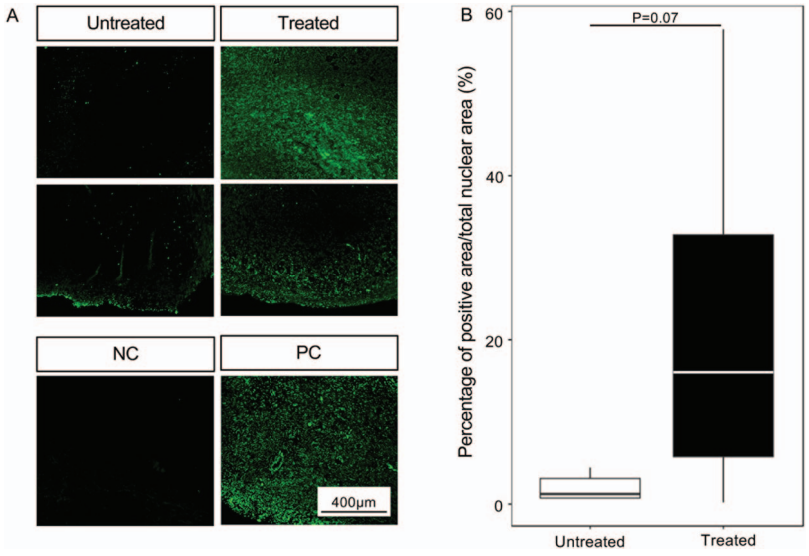

Figure 6 DNA fragmentation in ovarian stromal cells is influenced by first-line chemotherapy. (**A**) Representative images showing DNA damage in nuclei of stromal cells of untreated and treated patients. As positive control (PC), the tissue section was pre-treated with DNase I. As negative control (NC), no detection enzyme was used. (**B**) Quantification of TUNEL signal in stromal cell nuclei relative to the total nuclear area of the stained tissue sections of untreated ($n = 6$) and treated patients ($n = 7$).

also be caused by the chemotherapy-induced stromal damage that we observed.

Ovarian stromal cells

Analysis of cortical stroma revealed a significant increase in fibrotic area and an increase in DNA damage in treated compared to untreated patients. This finding is supported by other reports showing cortical fibrosis (Meirow *et al.*, 2007) and stromal apoptosis (Fabbri *et al.*, 2016) in ovarian tissue upon chemotherapy treatment. If stromal cells play a role in steroid production (as suggested by our steroid data), then fibrotic stroma might affect the quality of non-growing as well as early growing follicle.

Limitations

Our study has limitations. The study population was heterogeneous in terms of age and treatment exposures. Younger age might have contributed to the higher follicle atresia in the treated group. Furthermore, the impact of specific treatments could not be assessed. The limited tissue availability did not allow us to perform all the analyses in all patients and subsets had to be used, lowering the statistical power.

In summary, we demonstrated that exposure to first-line cancer treatment negatively impacts follicle number, follicle morphology, steroid secretion in culture and ovarian stromal compartment. Furthermore, we were able to show that atretic death in primordial follicles rather than ovarian 'burn-out' through follicle activation might be the dominant mechanism of action of ovarian failure following chemotherapy, as has also been suggested by others (Kerr *et al.*, 2012; Bedoschi *et al.*, 2016; Nguyen *et al.*, 2018; Winship *et al.*, 2018).

We observed a significant correlation between the density of atretic follicles and the cumulative exposure to alkylating agents and anthracyclines, which are widely used as first-line therapy in many pediatric solid cancers and hematological malignancies. The combined treatment with both drug types in the majority of the treated patients prevented identification of any drug-specific effects. Our observations suggest that OTC should be performed earlier, before the initiation of any chemotherapy, in order to prevent adverse effects on follicles and stroma. Follow-up investigations should focus on chemotherapy agent-specific effects on human follicles. In addition, assessing treatment effects on pre-pubertal primordial follicles as compared to adult primordial follicles should be considered.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

V.P. and M.W.: preparation of samples, experimental design, data collection and analysis and drafting the manuscript; B.A.A.: data collection and sample preparation; I.C.O. and M.S.: sample preparation; M.O.D.S. and J.L.: steroid analysis; O.H.: experimental design and interpretation of data; L.S.: experimental design, interpretation of data and drafting the manuscript; R.D.B.: performance of statistical analysis; M.O.:

preparation of samples; P.D.: experimental design, interpretation of data and drafting the manuscript; K.J.: experimental design, interpretation of data and drafting the manuscript. All authors approved the final article.

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Conflict of interest

None declared.

References

Abir R, Ben-Aharon I, Garor R, Yaniv I, Ash S, Stemmer SM, Ben-Haroush A, Freud E, Kravarusic D, Sapir O *et al.* Cryopreservation of in vitro matured oocytes in addition to ovarian tissue freezing for fertility preservation in paediatric female cancer patients before and after cancer therapy. *Hum Reprod* 2016;**31**:750–762.

Abir R, Ben-Haroush A, Felz C, Okon E, Raanani H, Orvieto R, Nitke S, Fisch B. Selection of patients before and after anticancer treatment for ovarian cryopreservation. *Hum Reprod* 2008;**23**:869–877.

Andersen CY, Ezcurra D. Human steroidogenesis: implications for controlled ovarian stimulation with exogenous gonadotropins. *Reprod Biol Endocrinol* 2014;**12**:128.

Anderson RA, Brewster DH, Wood R, Nowell S, Fischbacher C, Kelsey TW, Wallace WHB. The impact of cancer on subsequent chance of pregnancy: a population-based analysis. *Hum Reprod* 2018;**33**:1281–1290.

Asadi Azarbaijani B, Sheikhi M, Oskam IC, Nurmio M, Laine T, Tinkanen H, Makinen S, Tanbo TG, Hovatta O, Jahnukainen K. Effect of previous chemotherapy on the quality of cryopreserved human ovarian tissue in vitro. *PLoS One* 2015;**10**:e0133985.

Bedoschi G, Navarro PA, Oktay K. Chemotherapy-induced damage to ovary: mechanisms and clinical impact. *Future Oncol* 2016;**12**:2333–2344.

Demeestere I, Simon P, Dedeken L, Moffa F, Tsepelidis S, Brachet C, Delbaere A, Devreker F, Ferster A. Live birth after autograft of ovarian tissue cryopreserved during childhood. *Hum Reprod* 2015;**30**:2107–2109.

Donnez J, Dolmans MM. Transplantation of ovarian tissue. *Best Pract Res Clin Obstet Gynaecol* 2014;**28**:1188–1197.

Donnez J, Dolmans MM. Fertility preservation in women. *N Engl J Med* 2017;**377**:1657–1665.

Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, Ernst E, Luyckx V, Andersen CY. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. *Fertil Steril* 2013;**99**:1503–1513.

Duncan FE, Pavone ME, Gunn AH, Badawy S, Gracia C, Ginsberg JP, Lockart B, Gosiengfiao Y, Woodruff TK. Pediatric and teen ovarian tissue removed for cryopreservation contains follicles irrespective of age, disease diagnosis, treatment history, and specimen processing methods. *J Adolesc Young Adult Oncol* 2015;**4**:174–183.

El Issaoui M, Giorgione V, Mamsen LS, Rechnitzer C, Birkebaek N, Clausen N, Kelsey TW, Andersen CY. Effect of first line cancer treatment on the ovarian reserve and follicular density in girls under the age of 18 years. *Fertil Steril* 2016;**106**:1757–1762 e1751.

Ernst E, Kjaersgaard M, Birkebaek NH, Clausen N, Andersen CY. Case report: stimulation of puberty in a girl with chemo- and radiation therapy induced ovarian failure by transplantation of a small part of her frozen/thawed ovarian tissue. *Eur J Cancer* 2013;**49**:911–914.

Fabbri R, Macciocca M, Vicenti R, Paradisi R, Klinger FG, Pasquinelli G, Spisni E, Seracchioli R, Papi A. Doxorubicin and cisplatin induce apoptosis in ovarian stromal cells obtained from cryopreserved human ovarian tissue. *Future Oncol* 2016;**12**:1699–711.

Fabbri R, Vicenti R, Macciocca M, Pasquinelli G, Lima M, Parazza I, Magnani V, Venturoli S. Cryopreservation of ovarian tissue in pediatric patients. *Obstet Gynecol* 2012;**2012**:910698.

Familiari G, Caggiati A, Nottola SA, Ermini M, Di Benedetto MR, Motta PM. Ultrastructure of human ovarian primordial follicles after combination chemotherapy for Hodgkin's disease. *Hum Reprod* 1993;**8**:2080–2087.

Fowler PA, Anderson RA, Saunders PT, Kinnell H, Mason JI, Evans DB, Bhattacharya S, Flannigan S, Franks S, Monteiro A *et al.* Development of steroid signaling pathways during primordial follicle formation in the human fetal ovary. *J Clin Endocrinol Metab* 2011;**96**:1754–1762.

Gavish Z, Peer G, Roness H, Cohen Y, Meirow D. Follicle activation and 'burn-out' contribute to post-transplantation follicle loss in ovarian tissue grafts: the effect of graft thickness. *Hum Reprod* 2015;**30**:1003.

Gonfloni S, Di Tella L, Caldarola S, Cannata SM, Klinger FG, Di Bartolomeo C, Mattei M, Candi E, De Felici M, Melino G *et al.* Inhibition of the c-Abl-TAp63 pathway protects mouse oocytes from chemotherapy-induced death. *Nat Med* 2009;**15**:1179–1185.

Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev* 1996;**17**:121–155.

Green DM, Nolan VG, Goodman PJ, Whitton JA, Srivastava D, Leisenring WM, Neglia JP, Sklar CA, Kaste SC, Hudson MM *et al.* The cyclophosphamide equivalent dose as an approach for quantifying alkylating agent exposure: a report from the childhood cancer survivor study. *Pediatr Blood Cancer* 2014;**61**:53–67.

Hao J, Tuck AR, Sjodin MOD, Lindberg J, Sand A, Niklasson B, Argyraki M, Hovatta O, Damdimopoulou P. Resveratrol supports and alpha-naphthoflavone disrupts growth of human ovarian follicles in an in vitro tissue culture model. *Toxicol Appl Pharmacol* 2018;**338**:73–82.

Jadoul P, Dolmans MM, Donnez J. Fertility preservation in girls during childhood: is it feasible, efficient and safe and to whom should it be proposed? *Hum Reprod Update* 2010;**16**:617–630.

Jensen AK, Macklon KT, Fedder J, Ernst E, Humaidan P, Andersen CY. 86 successful births and 9 ongoing pregnancies worldwide in women transplanted with frozen-thawed ovarian tissue: focus on birth and perinatal outcome in 40 of these children. *J Assist Reprod Genet* 2017;**34**:325–336.

Ji J, Zhang Y, Redon CE, Reinhold WC, Chen AP, Fogli LK, Holbeck SL, Parchment RE, Hollingshead M, Tomaszewski JE *et al.* Phosphorylated fraction of H2AX as a measurement for DNA damage in cancer cells and potential applications of a novel assay. *PLoS One* 2017;**12**:e0171582.

Kalich-Philosoph L, Roness H, Carmely A, Fishel-Bartal M, Ligumsky H, Paglin S, Wolf I, Kanety H, Sredni B, Meirow D. Cyclophosphamide triggers follicle activation and "burnout"; AS101 prevents follicle loss and preserves fertility. *Sci Transl Med* 2013;**5**:185ra162.

Kerr JB, Hutt KJ, Michalak EM, Cook M, Vandenberg CJ, Liew SH, Bouillet P, Mills A, Scott CL, Findlay JK *et al.* DNA damage-induced primordial follicle oocyte apoptosis and loss of fertility require TAp63-mediated induction of Puma and Noxa. *Mol Cell* 2012;**48**:343–352.

Lande Y, Fisch B, Tsur A, Farhi J, Prag-Rosenberg R, Ben-Haroush A, Kessler-Icekson G, Zahalka MA, Ludeman SM, Abir R. Short-term exposure of human ovarian follicles to cyclophosphamide metabolites seems to promote follicular activation in vitro. *Reprod Biomed Online* 2017;**34**:104–114.

Lee SJ, Schover LR, Partridge AH, Patrizio P, Wallace WH, Hagerty K, Beck LN, Brennan LV, Oktay K, American Society of Clinical Oncology. American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. *J Clin Oncol* 2006;**24**:2917–2931.

Li J, Kawamura K, Cheng Y, Liu S, Klein C, Liu S, Duan EK, Hsueh AJ. Activation of dormant ovarian follicles to generate mature eggs. *Proc Natl Acad Sci U S A* 2010;**107**:10280–10284.

Lumley T. Dichromat: Color schemes for dichromats. 2013. https://CRAN.R-project.org/package=dichromat

Marcello MF, Nuciforo G, Romeo R, Di Dino G, Russo I, Russo A, Palumbo G, Schiliro G. Structural and ultrastructural study of the ovary in childhood leukemia after successful treatment. *Cancer* 1990;**66**:2099–2104.

Matthews SJ, Picton H, Ernst E, Andersen CY. Successful pregnancy in a woman previously suffering from beta-thalassemia following transplantation of ovarian tissue cryopreserved before puberty. *Minerva Ginecol* 2018;**70**:432–435.

McLaughlin M, Kelsey TW, Wallace WH, Anderson RA, Telfer EE. Non-growing follicle density is increased following adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) chemotherapy in the adult human ovary. *Hum Reprod* 2017;**32**:165–174.

Meirow D, Nugent D. The effects of radiotherapy and chemotherapy on female reproduction. *Hum Reprod Update* 2001;**7**:535–543.

Meirow D, Dor J, Kaufman B, Shrim A, Rabinovici J, Schiff E, Raanani H, Levron J, Fridman E. Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. *Hum Reprod* 2007;**22**:1626–33.

Nguyen QN, Zerafa N, Liew SH, Morgan FH, Strasser A, Scott CL, Findlay JK, Hickey M, Hutt KJ. Loss of PUMA protects the ovarian reserve during DNA-damaging chemotherapy and preserves fertility. *Cell Death Dis* 2018;**9**:618.

Oktay K, Harvey BE, Partridge AH, Quinn GP, Reinecke J, Taylor HS, Wallace WH, Wang ET, Loren AW. Fertility preservation in patients with cancer: ASCO clinical practice guideline update. *J Clin Oncol* 2018;**36**:1994–2001.

Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. *Cancer* 2007;**110**:2222–2229.

Petrillo SK, Desmeules P, Truong TQ, Devine PJ. Detection of DNA damage in oocytes of small ovarian follicles following phosphoramide mustard exposures of cultured rodent ovaries in vitro. *Toxicol Appl Pharmacol* 2011;**253**:94–102.

Poirot C, Abirached F, Prades M, Coussieu C, Bernaudin F, Piver P. Induction of puberty by autograft of cryopreserved ovarian tissue. *Lancet* 2012;**379**:588.

R Core Team. R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing, 2018. https://www.R-project.org

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998;**273**:5858–5868.

Roness H, Kashi O, Meirow D. Prevention of chemotherapy-induced ovarian damage. *Fertil Steril* 2016;**105**:20–29.

Rossi V, Lispi M, Longobardi S, Mattei M, Rella FD, Salustri A, De Felici M, Klinger FG. LH prevents cisplatin-induced apoptosis in oocytes and preserves female fertility in mouse. *Cell Death Differ* 2017;**24**:72–82.

RStudio Team. RStudio: Integrated Development for R. Boston, MA: RStudio, Inc., 2015. http://www.rstudio.com/

Shankar SM, Marina N, Hudson MM, Hodgson DC, Adams MJ, Landier W, Bhatia S, Meeske K, Chen MH, Kinahan KE *et al.* Monitoring for cardiovascular disease in survivors of childhood cancer: report from the cardiovascular disease task force of the Children's oncology group. *Pediatrics* 2008;**121**:e387–e396.

Tsai-Turton M, Luong BT, Tan Y, Luderer U. Cyclophosphamide-induced apoptosis in COV434 human granulosa cells involves oxidative stress and glutathione depletion. *Toxicol Sci* 2007;**98**:216–230.

van Dorp W, Haupt R, Anderson RA, Mulder RL, van den Heuvel-Eibrink MM, van Dulmen-den Broeder E, Su HI, Falck Winther J, Hudson MM, Levine JM *et al.* Reproductive function and outcomes in female survivors of childhood, adolescent, and young adult cancer: a review. *J Clin Oncol* 2018;**36**:2169–2180.

Wallace WH, Kelsey TW, Anderson RA. Fertility preservation in prepubertal girls with cancer: the role of ovarian tissue cryopreservation. *Fertil Steril* 2016;**105**:6–12.

Wallace WH, Smith AG, Kelsey TW, Edgar AE, Anderson RA. Fertility preservation for girls and young women with cancer: population-based validation of criteria for ovarian tissue cryopreservation. *Lancet Oncol* 2014;**15**:1129–1136.

Wallace WH, Thomson AB, Kelsey TW. The radiosensitivity of the human oocyte. *Hum Reprod* 2003;**18**:117–121.

Warnes GR, Bolker B, Bonebakker L, Gentleman R, Huber W, Liaw A, Lumley T, Maechler M, Magnusson A, Moeller S, Schwartz M, Venables B. Gplots: various R programming tools for plotting data. 2016. https://CRAN.R-project.org/package=gplots https://CRAN.R-project.org/package=gplots

Westergaard CG, Byskov AG, Andersen CY. Morphometric characteristics of the primordial to primary follicle transition in the human ovary in relation to age. *Hum Reprod* 2007;**22**:2225–2231.

Wickham H. Ggplot2: Elegant Graphics for Data Analysis. 2nd Edition. New York: Springer, 2009. https://doi.org/10.1007/978-0-387-98141-3

Wickham H. Reshaping data with the reshape package. *Journal of Statistical Software* 2007;**21**:1–20.

Winship AL, Stringer JM, Liew SH, Hutt KJ. The importance of DNA repair for maintaining oocyte quality in response to anti-cancer treatments, environmental toxins and maternal ageing. *Hum Reprod Update* 2018;**24**:119–134.