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Non-growing follicle density is increased followingadriamycin, bleomycin, vinblastine and dacarbazine (ABVD) chemotherapy in the adult human ovary

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Running Title: Chemotherapy and non-growing follicle density

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Abstract

Study question:Do the chemotherapeutic regimens of ABVD (adriamycin, bleomycin, vinblastine and dacarbazine), or OEPA-COPDAC (combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC)) used to treat Hodgkin lymphoma, affect the density, morphology and *in vitro* developmental potential of human ovarian follicles?

Summary answer: Ovarian tissue from women treated with ABVD contained a higher density of non-growing follicles/mm^3 and increased numbers of multi-ovular follicles, but showed reduced *in vitro* growth compared with patients with lymphoma who had not received chemotherapy, patients treated with OEPA-COPDAC, age-matched healthy women and age-related model-predicted values.

What is known already: Chemotherapy regimens can cause a loss of follicles within the ovary that depends on the drugs given. Early stage Hodgkin lymphoma is commonly treated by ABVD, a non-alkylating regimen which apparently has ovarian sparing qualities,thus it is important to investigate the histological appearance and distribution of follicles within ABVD-treated ovarian tissue.

Study design, size, duration: Thirteenovarian biopsies were obtained from Hodgkin lymphoma patients (6 adolescents and 7 adults)and one biopsy from a non-Hodgkin lymphoma patient.TwoHodgkin lymphoma patients and the non-Hodgkin lymphoma patient had received no treatment prior to biopsy collection. The remaining 11 Hodgkin lymphoma patients received one of two regimens; ABVD or OEPA-

COPDAC. Tissue was analysed histologically andcompared to biopsies from healthy women, and in a sub-group of patients, tissue wascultured for 6 days *in vitro.*

Participants/materials, setting, methods: Ovarian biopsies were obtained from patients undergoing ovarian cryopreservation for fertility preservation, and from healthy women at the time of Caesarian section ('obstetric tissue'). Follicle number and maturity were evaluated in sections of ovarian cortical tissue, and compared to an age-related model of mean follicle density and to age-matched contemporaneous biopsies. The developmental potential of follicles was investigated after 6 days tissue culture.

Main results and the role of chance: A total of 6877 follicles was analysed.ABVD-treated tissue contained a higher density of non-growing follicles/mm^3 (230±17) (mean±SEM) than untreated (110±54), OEPA-COPDAC-treated (50±27 and obstetric tissue (20±4)(P< 0.01),with follicle density 9-21 standard deviations higher than predicted by an age-related model. Bi-ovular and binucleated non-growing follicles occurred frequently in ABVD-treated and in adolescent untreated tissue but were not observed in OEPA-COPDAC-treated or obstetric tissue, although OEPA-COPDAC-treated tissue contained a high proportion of morphologically abnormal oocytes (52% versus 23% in untreated, 22% in ABVD-treated and 25% in obstetric tissue; P< 0.001). Activation of follicle growth *in vitro*occurred in all groups, but in ABVD-treated samples there was very limited development to the secondary stage, whilst in untreated samples from lymphoma patients growth was similar to that observed in obstetric tissue (untreated; P< 0.01 versus ABVD-treated, ns versus obstetric).

Large scale data: N/A

Limitations, reasons for caution: Although a large number of follicles were analysed, these data were derived from a small number of biopsies. The mechanisms underpinning these observations have yet to be determined and it is unclear how they relate to future fertility.

Wider implications of the findings: This study confirmsthat the numberof non-growing follicles is not depleted following ABVD treatment, consistent with clinical data that female fertility is preserved. Our findings demonstrate that immature follicle density can increase as well as decrease following at least one chemotherapy treatment. This is the first report of morphological and follicle developmental similarities between ABVD-treated tissue and the immature human ovary. Further experiments will investigate the basis for the marked increase in follicle density in ABVD-treated tissue.

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Keywords: ovary/ follicle/ lymphoma/
ABVD/oocyte/primordial/chemotherapy/regeneration/ modelling

Introduction

The number of follicles in the ovary decreases progressively with age. Treatments such as some chemotherapy agents can accelerate this loss leading to premature ovarian insufficiency (POI) with loss of fertility and estrogen deficiency in female survivors of cancer (Morgan *et al*., 2012). The likely range of mechanisms of drug-induced ovarian damage hasyet to be fully characterised but includes direct damage to growing and non-growing follicles, and loss of growing follicles may lead to the recruitment of dormant follicles to the growing phase which in turn are lost, thus accelerating the depletionof the non-growing pool, ie the ovarian reserve(Meirow *et al*., 2010; Morgan *et al*., 2012; Kalich-Philosoph *et al*., 2013).

Since the mid-1970s the standard first line treatment for early stage Hodgkin lymphoma (HL)in many countries has been adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) (Bonadonna and Santoro, 1982; Meirow and Nugent, 2001; Meyer *et al*., 2012). Early stage HL patients receiving ABVD based treatment where involved field radiotherapy does not include the ovarieshave a good reproductive prognosis for both fertility and risk of POI (Hodgson *et al*., 2007,Swerdlow *et al*., 2014,).In contrast advanced stage HL patients treated with a drug regimen containing one or more alkylating agents and involved field radiotherapy have a higher prevalence of POI (Behringer *et al*., 2005), and should therefore be considered for fertility preservation strategies before commencing treatment (Anderson *et al*., 2015).

The purpose of this study was to examinemicroscopically, ovarian tissue from patients with lymphoma exposed to ABVD chemotherapy and compare this with samples exposed to the combined alkylating chemotherapeutic regime (OEPA-

COPDAC)(combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC), with samples not previously exposed to chemotherapy and with further samples from healthy women to determine whether follicle density, morphology and *in vitro* developmental potential were affected by these chemotherapeutic interventions. Comparing follicle densities across treatments requires identification of changes to non growing follicle(NGF) cortical densities in comparison with those predicted by age related models (Kelsey & Wallace., 2012; Kelsey et al., 2013).In this study we used a recently reported validated age-related model of mean follicle density(MFD) in the ovarian cortex (McLaughlin *et al*., 2015), to compare observations of MFD in tissue obtained after different chemotherapy regimens.

Materials and Methods

Patient Selection

Diagnosis, patient age, chemotherapeutic regimen, anti-Müllerian hormone (AMH) concentrationsand time between completion of treatment and biopsy collection are detailed in Table 1.

Ovarian Cortical Tissue

Ovarian biopsies were obtained laparoscopically from 6 adolescentsand 7 adults diagnosed with Hodgkin lymphoma and 1 adult diagnosed with non-Hodgkin lymphoma. All patients were undergoing removal of ovarian cortex for fertility cryopreservation either prior to chemotherapy or following relapse of previously treated illness. Protocols for tissue donation for research had Ethical Committee approval fromSouth East Scotland Research Ethics Committee (ref 06/S1103/26) and all patients gave informed consent in writing. The mean patient age was

20.2±1.5 years (mean ±SEM) with a range of 12.0 – 30.0 years. For analyses patients were divided into 3 groups: those treated with ABVD (aged 16 – 29 years, *n* = 8), those treated with OEPA-COPDAC (aged 14 – 16 years, *n* = 3) and untreated patients or controls (aged 12 – 30 years, *n* = 3). Data were compared with results obtained from contemporaneous ovarian biopsies obtained from adult women undergoing elective Caesarean section (age range 23 – 39 years, *n* = 12) prepared and processed in an identical manner, also obtained with written informed consent and Ethical committee approval (ref 10/S1101/24).

Tissue preparation and processing

Fresh ovarian biopsies(ranging in size from 8x5mm and 6x4mm all with variable thickness)were transported to the laboratory in holding medium (Leibovitz medium, Gibco; Life Technologies, Paisley, Renfrewshire, UK supplemented with 2mM sodium pyruvate, 2mM L-glutamine, 3mg/ml human serum albumin, 75mg/ml penicillin G and 50mg/ml streptomycin; all chemicals from Sigma-Aldrich, Poole, UK). Tissue was transferred into fresh pre-warmed (37^{o}C) holding medium and examined under a dissecting light microscope. A scalpel and fine forceps were used to remove any damaged or haemorrhagic areas as well as any tissue adhering to the underside of the biopsies to leave only intact cortex in place. Using a scalpel the tissue was divided into fragments of approximately 4 x 2 x 0.5 mm; the number of fragments varied between biopsies. Fragments were fixed in 10% neutral buffered formalin (NBF) for 48h then processed and prepared for staining and microscopic evaluation as previously described (McLaughlin *et al*., 2014).

Thawing of cryopreserved tissue

Two of the biopsies were cryopreserved by slow freezing (Gosden *et al*., 1994) and were donated for research at the patients' request. Tissue was thawed as described previously (Anderson *et al*., 2014), inspected, divided into fragments, fixed and processed for staining and analysis as described above.

Assessment of mean follicle density in ovarian biopsies

Each histological section of every tissue fragment was examined under light microscopy. Follicles were categorised according to their stage of development as previously described (Telfer *et al*., 2008; McLaughlin *et al*., 2014). To avoid over-counting, only follicles containing the nucleolus were assessed. The volume of tissue analysed per patient was calculated as described previously (Lass *et al*., 1997; Anderson *et al*., 2014). Briefly, tissue volume was calculated as the sum of the area in mm^2 of all tissue sections analyzed per patient, multiplied by 0.006 mm (the thickness of the sections) to give a value in mm^3. The area of each section was measured andmean follicle density was determined by dividing the total number of follicles per patient by the volume of tissue analyzed in mm^3 (Anderson *et al*., 2014).

Evaluation of histology

Evaluation of the tissue sections was performed blinded to the treatment groups. Follicles in freshly fixed and cultured tissue pieces were categorised by developmental stage based on morphology as previously described (McLaughlin *et al*., 2014).Follicle morphological normality was determined by an assessment of the appearance of the oocyte and surrounding cells using the cross-section containing the nucleolus as described previously (McLaughlin and Telfer, 2010, McLaughlin *et al*., 2014). The presence of bi-ovular and binucleate follicles was also noted.

The spatial relationship between follicles was also assessed and classified as (1) single i.e. no follicles within 15 μm of the follicle being evaluated, (2) in close proximity i.e. at least one other follicle occurring within 15μm of the follicle being evaluated or (3) direct contact i.e. where 2 or more follicles appeared to share the same basal lamina or the basal laminae of 2 or more follicles abutted. Follicles were classified as occurring in clusters if 5 or more follicles were found in direct contact or close proximity. The presence of naked oocytes with few or no surrounding cells was also noted.

Fragment culture

Prior to fixation a number of tissue fragments were selected at random from a sub-section of the ABVD-treated patients (*n* = 4; age range 23 - 29 years), 1 OEPA-COPDAC-treated patient (16 years), 2 untreated controls (15 years and 30 years) and from obstetric patients (*n* = 10; age range 23 – 36 years). Between 2 and 6 fragments were obtained for each patient, prepared for culture and incubated for 6 days then processed for histological evaluation as previously described (McLaughlin *et al*., 2014).

Immunohistochemistry

A number of ABVD-treated, OEPA-COPDAC-treated and control tissue fragments fixed in NBF and embedded in paraffin as described previously (McLaughlin *et al*., 2014) were selected at random and cut in 6μm sections and mounted on charged slides to investigate the expression of the germline marker proteinDEAD box polypeptide 4 (DDX4). Antigen retrieval was performed using 0.01M sodium citrate and endogenous peroxidase activity quenched by 3% hydrogen peroxide in

methanol. Tissue sections were incubated in anti-DDX4/MVH ab13840 polyclonal primary antibody (Abcam, Cambridge, UK) overnight at 4^{o}C. Negative controls were established by replacing the primary antibody with goat serum. On completion of incubation, sections were washed and probed with anti-rabbit secondary antibody labelled with horseradish peroxidase for 30 mins (ABC-Elite Rabbit IgG, Vectastain Elite Kit, PK-6101, Vector Laboratories Ltd, Peterborough, UK). DDX4 was detected using 3, 3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories Ltd, Peterborough, UK). Structures were positively identified as germ cells when brown staining was observed within a cell.

Statistical analysis

Observed mean follicle densities were compared to predicted mean follicle densities using an age-related model of mean follicle density in the cortex of the human ovary (McLaughlin *et al*., 2015). Chi-Square Goodness-of-Fit analysis was used to test the null hypotheses that the observations matched predicted MFD. Pearson's product-moment correlation coefficients were also calculated tocompare datato the line of identity where predictions exactly match observations. Due to the need for high confidence in the significance of any observed differences between predicted and observed densities, statistical significance was set at the 99% level throughout. Bland-Altman analysis was used to estimate the number of standard deviations away from agreement between observations and predictions using the limits of agreement reported in McLaughlin *et al*. (2015).

Results

Follicle density

The number of follicles and their developmental stage were determined by examining fresh and post-thawed fixed ovarian tissue from 14 previously treated and untreated lymphoma patients and an age-matched group of 12 obstetric patients; a total of 6877 follicles were examined. The total number of follicles counted in each group and the volume of tissue analysed are shown in Table 2. Ages and MFDs for the three groups of lymphoma patients are shown in Fig. 1A, together with age-related predictions taken from McLaughlin *et al*., (2015). For the three patients whoreceived no chemotherapy we have insufficient evidence to reject the null hypothesis that the observed values for MFD are a perfect fit with predicted values taken from the age-related model derived from data from other subjects receiving no chemotherapy: Chi-square p-value = 0.07; Pearson's product-moment correlation coefficient = 0.999 (p< 0.01, Fig. 1B) with the observations within 1.25 standard deviations of agreement between observed and predicted values (Fig. 1C). Thethreepatients who received OEPA-COPDAC chemotherapy had significantly lowerobserved MFD than predicted: Chi-square p-value < 0.01; Pearson's product-moment correlation coefficient = -0.399 (p = 0.74, Fig. 1B) with the observations between 0 and 11 standard deviations lower than the predictions (Fig. 1C).

However,the eight patients who had received ABVD chemotherapy had higher numbers of non-growing follicles (160-303/mm^3) compared with samples from healthy women (8-46/mm^3) (Table 2). The ABVD group had significantly higherobserved than predicted MFD: Chi-square p-value < 0.001; Pearson's product-moment correlation coefficient = 1.57 (p = 0.71, Fig. 1B) with observations between 9 and 21 standard deviations higher than the predictions (Fig.1C).

Time elapsed between completion of chemotherapy regimen and biopsy ranged from 1 to 36 months, with six of the eight ABVD-treated patients having tissue collected within 6 weeks and all OEPA-COPDAC patients undergoing biopsy within 9 months of treatment. No correlation was foundbetween the time interval and the MDF observed in tissue treated with either regimen: the marked increase in MFD in ABVD-treated tissues was observed in biopsies taken within 4 weeks of chemotherapy completion and was also present in samples taken up to 36 months after treatment, although the small sample size at the later treatment times does not allow for time after treatment to be robustly tested. The OEPA-COPDAC tissue from two out of three patients showed follicle numbers below that predicted at 6-9 months post treatment.Biopsy tissue was only available from each patient at one time point therefore it was not possible to investigate by histological means whether deterioration or recovery of NGF density occurred over time.

Follicle categorisation

The majority of all follicles observed in fixed tissue were non-growing irrespective of illness, treatment or age (Fig. 2A). However ABVD-treated tissue contained a significantly smaller percentage of growing follicles (3.0%) compared to untreated, OEPA-COPDAC-treated and obstetric patients (15.6%, 21.4% and 18.0% respectively; $p< 0.001$) (Fig. 2A).

Analysis of oocyte appearance showed that in biopsies collected from untreated, ABVD-treated and obstetric patients, morphological normality was high with >74% of oocytes appearing normal. In contrast less than half of the oocytes in follicles observed in OEPA-COPDAC-treated tissue met the criteria for normality (41.9% in

OEPA-COPDAC compared to 76.8% in untreated; 78.4% in ABVD-treated and 74.2% in obstetric samples; $p< 0.001$) (Fig. 2C and D).

Follicle development in cultured tissue

To investigate the *in vitro* developmental potential of chemotherapy-exposed follicles, tissue fragments from a subset of patients representing each group were cultured for 6 days. It was not possible to culture fragments from everypatient due to the limited amount available. A total of 89, 614 and 274 follicles were analysed in cultured tissue obtained from untreated (n = 2), ABVD-treated (n = 4) and obstetric patients (n = 10) respectively. Analysis of cultured tissue fragments from two patients treated with OEPA-COPDAC has been omitted due to extreme follicle degeneration in both patients. Initiation of follicle growth was observed in all of the 3 remaining groups. Post-culture the proportion of growing follicles was 19.9% in ABVD-treated tissue, 41.6% in untreated tissue and 46.3% in obstetric samples (all $P< 0.001$ versus uncultured)(Fig. 3A). Development to the secondary stage occurred in both untreated and obstetric groups with 18% and 18.2% of follicles observed reaching this stage respectively whereas very few follicles progressed in the ABVD-treated tissue,comprisingonly 1.2% of follicles at the secondary stage after culture (Fig 3B and C).

Immunohistochemical localization of DDX4

Due to the high density of non-growing follicles observed in ABVD-treated tissue and the presence of clusters, bi-ovular and binucleate follicles, immunohistochemistry was performed to examine whether the germline marker DDX4 could be localized in these structures. Uncultured tissues sections from 9 patients representing all groups

were studied; the number of sections per patientwas variable due to tissue availability, and the number of follicles per section was also highly variable. Discrete positive DDX4 staining was observed in oocytes of follicles at all stages of development in all groups. DDX4 was also localised to the bi-ovular and binucleate oocytes and clusters of naked oocytes with adjacent or shared oolemmae observed in ABVD-treated tissue. No positive staining was observed in any tissue sections where the primary antibody had been omitted (Fig. 3D).

Spatial distribution of follicles

In all groups individual follicles and groups of follicles were distributed unevenly throughout the cortex however the pattern of distribution varied between groups with the majority of follicles occurring discretely (single follicles ≥ 15μm apart)in obstetric tissue (Fig. 4A and B). ABVD-treated and untreated adolescent tissues appearedmarkedly different from the others examined, withsignificantly fewer discrete follicles seen and clusters of closely packed follicles (5 or more NGF ≤ 15μm apart) observed in these groups($p < 0.05$; ABVD-treated and untreated versus OEPA-COPDAC-treated and obstetric patients(Fig.4A and C).Bi-ovular and binucleate follicles were observedin ABVD-treated tissues and also in the 12 year old untreated patient's tissue comprising between 8-18% of the NGF population. These structures were not observed in the other tissues examined. Follicle clusters often contained naked or partially naked oocytes, which otherwise appeared morphologically normal (Fig 4C).

Discussion

Ovarian dysfunction and reduced fertility potential can be a consequence of cytotoxic therapy particularly where alkylating agents have been used, with resultant loss of the ovarian reserve (Meirow *et al*., 2010). In contrast women diagnosed with lymphoma and treated with the gonad-sparing regimen ABVD have a low risk of significant impairment of fertility (Hodgson *et al*., 2007)or of POI (Swerdlow *et al*., 2014) and no reduction in follicle numbers have been observed (Seshadri et al., 2006). The impact of chemotherapy on the ovarian reserve is evaluated indirectly by clinical parameters including amenorrhea,AMH and FSH levels (Meirow, 2000; Oktay *et al*., 2006; Anderson and Cameron, 2011). Post-treatment AMH levels in lymphoma patients clearly show the differential impact of ABVD and alkylating agent based regimens (Decanter *et al*., 2010)and AMH levels may also be reduced at the time of diagnosis (Lawrenz *et al*., 2012). Although early studies showed that chemotherapy reduced the number of antral follicles in the ovary in girls treated for leukaemia (Himelstein-Braw *et al*., 1978),this is the most detailed quantitative analysis of the direct effect of chemotherapy treatment on density of the ovarian reserve in lymphoma patientsand the impact of the different chemotherapy regimens on morphology and *in vitro* developmental potential of follicles. In this study we have identified and quantified differences in the non-growing follicle population of treated and untreated lymphoma patients. The observed MFD of untreated lymphoma patients were close to the densities predicted by an age-related model indicating that the disease itself is not implicated in the variationsof the ovarian reserve observed in the chemotherapy-treated groups.

Treatment regimens containing alkylating agents such as cyclophosphamide are known to lead to POI via a direct or indirect reduction in the NGF population (Oktem and Oktay, 2007; Meirow, 2000; Meirow *et al*., 2010).Our finding that patients treated

with the OEPA-COPDAC regimen had lower than predicted MFDs confirms and quantifies this, although the MFD in this small group varied from very low to close to average for age. Further analysis of the degree of loss is not possible without knowing pre-treatment values. In this study patients treated with OEPA-COPDAC were all teenagers (14, 15 and 16 years) at the time of tissue collection, which was 6-9 months after completion of chemotherapy. Of the 3 patients included in this study treated with OEPA-COPDAC one was diagnosed with reduced ovarian reserve based on having regular menstrual cycles but undetectable AMH levels one year after completion of treatment and another was diagnosed with POI 6 years post chemotherapy.

Surprisingly we found a striking and statistically highly significant increase in follicle number and MFD after ABVD chemotherapy. All eight ABVD patients had higher follicle counts (Table 2) and a markedly higher follicle density than predicted (Fig. 2C).Despite the well reported variation of follicle density between and within human cortical biopsies follicles (Kohl *et al*., 2000; Qu *et al*., 2000; Poirot *et al*., 2002; Schmidt *et al*., 2003; McLaughlin *et al*., 2015), all ABVD-treated samples consistently showed an increase in the NGF population whereas this was not seen for any of the other groups or individual biopsies. We initially considered that thisincreasemight be attributed to a reduction in ovarian volume during treatment as all treatments would result in an initial reduction in ovarian volume because of the loss of large follicles.However, MFD is based on volume of ovarian cortex, not whole ovary and there is no evidence to support a differential effect on ovarian cortex volume by any treatment.Additionally, these samples were collected 4 weeks to 36 months after the completion of treatment with resumption of follicular growth (indicated by regular menses) in those with longer intervals and there was no apparent relationship

between increased follicle number/MFD and interval since treatment. The observed differences between ABVD MFD and control data is between 9-21 standard deviations (Figure 1C) and so a reduction in cortical volume would need to be of that order of magnitude. There are no clinical data/observations to support such a degree of shrinkage.

The underlying mechanism for an increase in MFD after ABVD treatment remains to be established. An alternative explanation may be that this treatment has resulted in new oocytes/follicles being formed. Putative germline stem cells or oogonial stem cells, which may be capable of regenerating the NGF population under perturbed conditionshave been identified within the adult human ovary (White *et al*.,2012;Dunlop *et al*., 2013;Hanna and Hennebold, 2014). It is possible that the ABVD combination or specific components activate these cells to form oocytes or oocyte like structures. Recent studies have shown that mesenchymal stem cells are sensitive to bleomycin treatment (Nicolay et al., 2016) but nothing is known about how these drugs affect putative germline stem cells and other ovarian cells.

We also observed that post-ABVD tissue contains follicle clusters, many containing bi-ovular and binucleate follicles, a feature more commonly associated with the prepubertal ovary (Anderson et al., 2014). Follicles with more than one oocyte are reported during fetal development and in early life in many mammalian species (Papadaki, 1978; Telfer and Gosden 1987; Silva-Santos and Seneda, 2011) but are much rarer in adults (Turkalj *et al*., 2013) and may be preferentially lost with abnormal follicles during childhood (Anderson *et al*., 2014). No bi-ovular or binucleate follicles were observed in tissue from healthy women,or following OEPA-COPDAC treatment, highlighting that this difference inNGF patterning in the cortex of tissue is a result of the specific chemotherapy treatment. Furthermore, the *in vitro*

developmental potential of ABVD-treated tissue showed differences compared to control tissue, with limited follicle development, comparable to that from prepubertal girls(Anderson *et al*., 2014).We suggest that the lack of development in ABVD-treated tissue may be attributed to theinhibitory effect exerted by the high density of primordial follicles present (Da Silva-Buttkus *et al*., 2009). Initiation of follicle growth was observed in the obstetric patient cohort supporting the findings of previous studies (Anderson*et al*., 2014, McLaughlin *et al*., 2014).

In summary this study demonstrates that ABVD treatment does not diminish the ovarian reserve and may paradoxically increase it. Other significant features such as the presence of bi-ovular and binucleate oocytes and clustering are increased. In this respect, ABVD-exposed tissue is similar to that from prepubertal girls, and this similarity is also reflected in its reduced capacity for follicle development *in vitro*. The number of patients analysed is small and so interpretation should be cautious. However, the data presented here have highlighted a phenomenon that has not been previously reported.Further investigation is required to elucidate the mechanism by which the ovarian reserve is affected by ABVD treatment, and the consequences for later fertility and reproductive lifespan.

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Authors Roles:

MM[c] Preparation of samples, experimental design, data collection and analysis, drafting the manuscript and approval of final version.

TWK Data analysis and interpretation, drafting the manuscript and approval of final version.

WHBW Acquisition of clinical data and interpretation,drafting the manuscript and approval of final version.

RAA Sample collection, acquisition of data and interpretation,drafting the manuscript and approval of final version.

EET Experimental design, analysis and interpretation,drafting the manuscript and approval of final version.

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Conflict of Interest

The authors have no conflicts of interest to declare

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Legends to Figures

Figure 1 (A). Observed and predicted human mean follicle density (MFD). Black dots represent model-predicted values from ages 10 through 50 years. Blue dots denote MFDs of patients that did not receive chemotherapy, green dots patients that received OEPA-COPDAC(combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC)) and red dots patients that receivedABVD (adriamycin, bleomycin, vinblastine and dacarbazine). All tissue was uncultured. **(B)** Pearson product-moment correlation. The line of identity represents the idealised confluence of observed and predicted values. Blue dots indicate observed against predicted MFD for 3 untreated patients. **C)** Bland-Altman Plot. The x-axis represents means (i.e. half the sum of observed and predicted MFD values); the y-axis represents the differences (i.e. predicted MFD values subtracted from observed values). The solid horizontal line shows no trend between means and differences. Blue dots denote difference from predicted MFD for untreated patients; these are between 0 and 1.25 standard deviations (i.e. between 0 and 16 follicles) below the zero predicted difference. Greens dots denote difference from predicted MFD for 3 OEPA-COPDAC-treated patients' chemotherapy protocol; these are between 1 and 11 standard deviations (i.e. between 12 and 132 follicles) below the zero predicted difference. The red dots denote difference from predicted MFD for 8 ABVD-treated patients; these are between 9 and 21 standard deviations (i.e. between 108 and 250 follicles) above the zero predicted difference.

Figure 2 (A) Distribution of follicle classes (as percentage of total) in fixed ovarian tissue from either untreated,OEPA-COPDAC (combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC))or ABVD (adriamycin, bleomycin, vinblastine and

633 dacarbazine)treated girls and adults, and obstetric patients. 469, 903, 5001 and 504
634 follicles were classified in the four groups, respectively. Blue: non-growing follicles;
635 red: primary follicles; green: secondary follicles. (**B**) Photomicrographs of non-
636 growing follicles in a 22 year-old lymphoma patient treated with ABVD (main image
637 and inset). Scale bars 30μm inset and 50μm main image.**(C)** Assessment of oocyte
638 appearance within growing and non-growing follicles in fixed tissue. Fewer
639 morphologically normal oocytes were observed in OEPA-COPDAC-treated tissue
640 compared to other groups (p<0.001) (**D**) Photomicrograph of morphologically
641 abnormal follicles in fixed tissue donated by a 16 year-old OEPA-COPDAC-treated
642 patient (main image and inset).Scale bars 30μm inset and 50μm main image.
643 **Figure 3(A)**. Distribution of follicle classes (as percentage of total) in ovarian tissue
644 cultured for 6 days from untreated (n=2) and ABVD (adriamycin, bleomycin,
645 vinblastine and dacarbazine) treated girls and adults (n=4), and obstetric patients
646 (n=10). Blue: non-growing follicles; red: primary follicles; green: secondary follicles. A
647 total of 89, 614 and 274 follicles are classified in the three groups, respectively. **(B)**
648 Photomicrograph of *in vitro* activated follicles in 23 year-old obstetric tissue. Scale
649 bar 60μm. **(C)** Photomicrograph of non-growing follicles in 23 year-old ABVD-treated
650 tissue after incubation for 6 days. Scale bar 60μm.**(D)** Photomicrograph showing
651 immunohistochemical detection of DDX4 in 22 year old ABVD-treated ovarian cortex.
652 (i) Brown staining indicating present in all structures morphologically identified as
653 germ cells. (ii) Negative control where primary antibody was omitted. Scale bar =
654 60μm.
655 **Figure 4 (A)**. Incidence (i.e.number of observations) of single (blue) and clustered
656 (red) follicles as percentage of total in fixed ovarian tissue from untreated, OEPA-
657 COPDAC(combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and

cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC)) treated, ABVD (adriamycin, bleomycin, vinblastine and dacarbazine)-treated girls and adults, and obstetric patients. **(B)** Photomicrograph ofnon-growing follicles(NGFs) in fixed tissue from 36 year-old obstetric tissue. Dashed blue lines indicating a distance of ≤15μmbetween follicles. Scale bar 100μm. **(C)** Photomicrograph of non-growing follicles in fixed tissue 22 year-old ABVD-treated tissue. Purple circles indicate areas of clustered NGFs within 15μm or less of each other. Scale bar = 60μm. Inset: non-growing bi-ovular follicle in ABVD-treated tissue. Scale bar 25μm in inset

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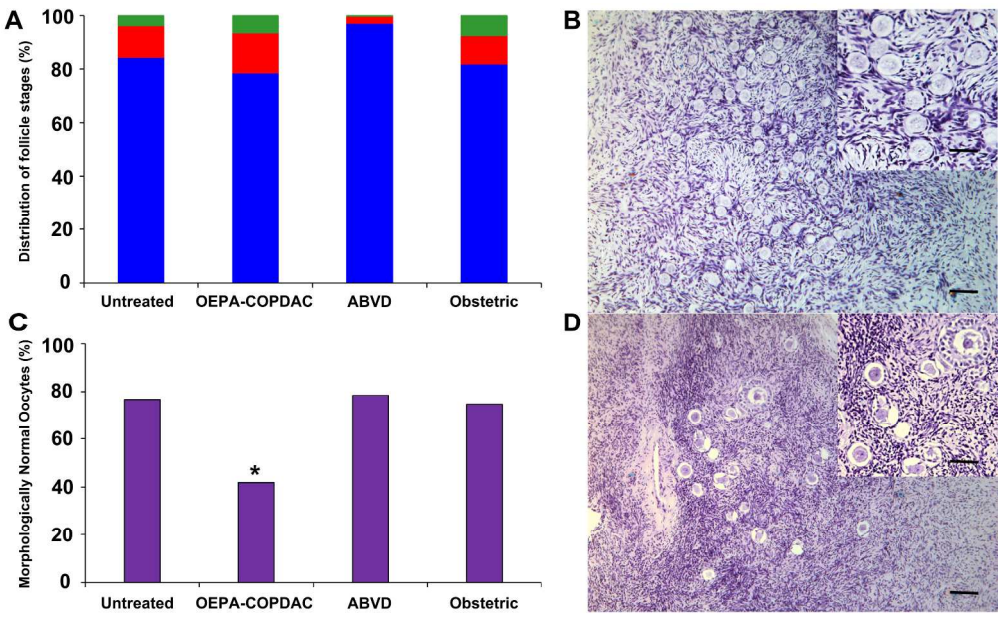

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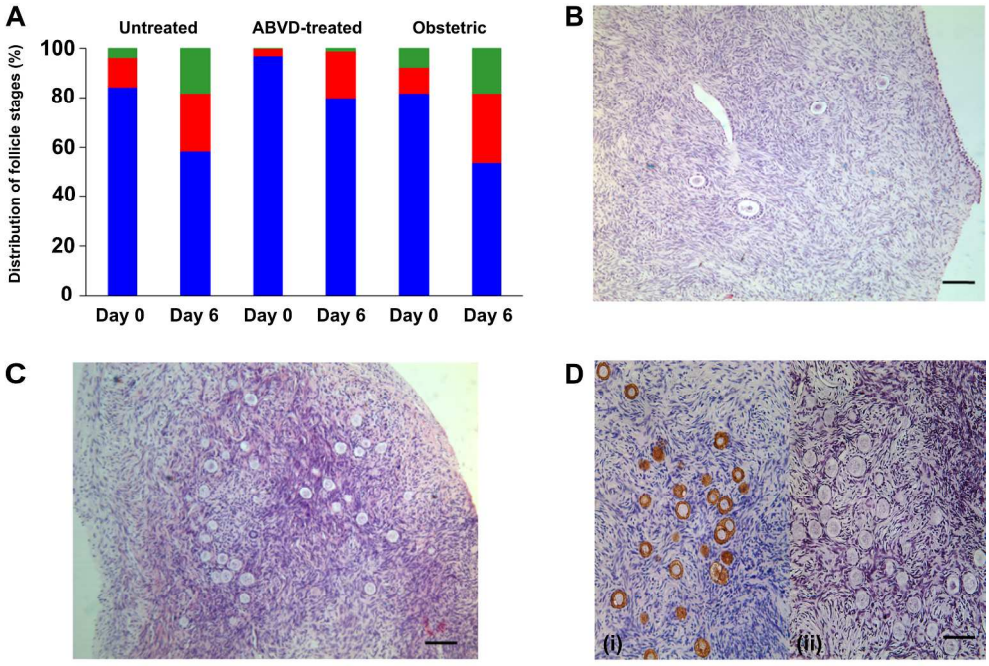

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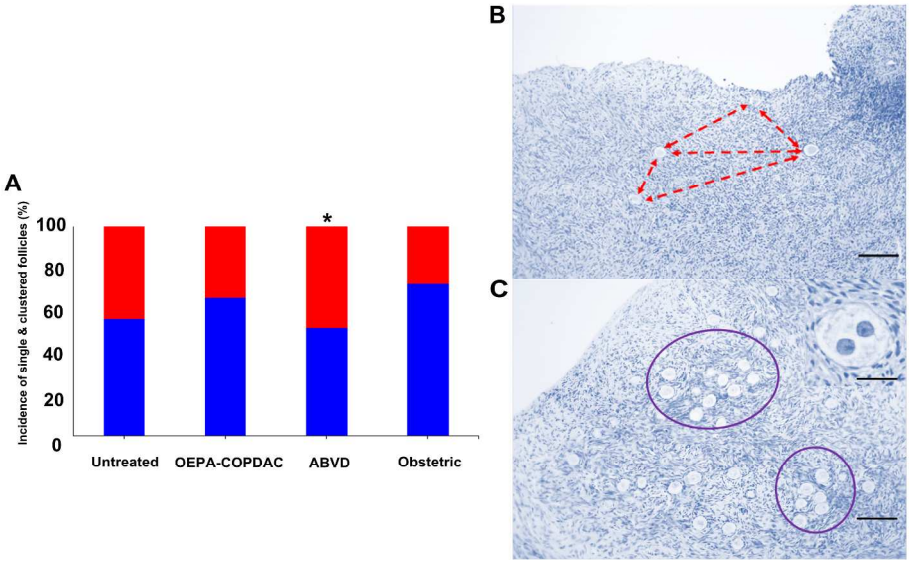

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Table 1. Treatment regimen (ABVD (adriamycin, bleomycin, vinblastine and dacarbazine), or OEPA-COPDAC (combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC)), anti-Müllerian hormone (AMH) level and treatment to biopsy time interval of patients contributing tissue. All had Hodgkin's lymphoma except the final one (aged 30) who had non-Hodgkin's lymphoma.

Age (years)	Treatment	AMH (pmol/L)	Treatment to Biopsy Interval
12	none	<4	n/a
14	OEPA COPDAC	not taken	9 months
15	OEPA COPDAC	not taken	6 months
15	none	<4	n/a
16	OEPA COPDAC	10.1	6 months
16	ABVD	<4	4 weeks
21	ABVD	not taken	4 weeks
22	ABVD	<4	4 weeks
22	ABVD	<4	6 months
23	ABVD	<4	4 weeks
23	ABVD	<4	4 weeks
25	ABVD	<4	36 months
29	ABVD	not taken	6 weeks
30	none	not taken	n/a

Table. 2. Number of follicles and non-growing follicles (NGFs) analysed in ovarian cortical tissue of Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL) and obstetric patients undergoing elective caesarean section (ECS). HL and NHL patients either received adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) or combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC) OEPA-COPDAC) or no treatment (Nil) prior to biopsy collection.

Diagnosis	Age (Years)	Treatment	Vol mm^3 Analysed	Total No. follicles	Total No. NGFs	NGFs/mm^3
HL	12	Nil	0.92	195	187	203
HL	15	Nil	1.7	259	194	114
NHL	30	Nil	0.9	15	15	17
HL	14	OEPA-COPDAC	1.8	19	19	10.5
HL	15	OEPA-COPDAC	5.84	732	605	103
HL	16	OEPA-COPDAC	2.14	152	86	40
HL	16	ABVD	4.75	988	988	208
HL	21	ABVD	1.53	473	464	303
HL	22	ABVD	2.66	605	599	225
HL	22	ABVD	1.64	444	437	266
HL	23	ABVD	2.41	712	660	273
HL	23	ABVD	2.6	558	506	194
HL	25	ABVD	3.06	642	617	201
HL	29	ABVD	3.62	579	578	160
ECS	23	N/A	1.21	38	32	26
ECS	24	N/A	1.45	85	73	50
ECS	26	N/A	2.01	77	63	32
ECS	28	N/A	2.28	67	51	22
ECS	28	N/A	1.06	60	49	46
ECS	31	N/A	2.72	39	38	14
ECS	33	N/A	2.31	34	29	12.5
ECS	33	N/A	1.98	36	26	13
ECS	36	N/A	1.55	17	14	9
ECS	36	N/A	2.39	15	15	6
ECS	36	N/A	2.71	22	12	4
ECS	39	N/A	1.32	14	11	8