**Running title:** Impact of gonadotoxic pre-treatment

# The effect of first line cancer treatment on the ovarian reserve and follicular density in girls under the age of 18 years

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**Capsule**

The ovarian reserve is moderately reduced by first-line gonadotoxic treatment in young cancer patients under the age of 18 years requiring fertility preservation prior to treatment of relapse.

**Abstract**

*Objective:* To study the impact of first-line antineoplastic treatment on the ovarian reserve in young girls returning for ovarian tissue cryopreservation (OTC) in connection with a relapse.

*Design:* Retrospective case-control study.

*Setting:* Two university hospitals.

*Patients:* 63 girls under the age of 18 years who underwent OTC before (group 1: 31 patients) and after (group 2: 32 patients) initial cancer treatment.

*Intervention(s):* None.

*Main Outcome Measure(s):* Follicular densities (follicles/$mm^3$) measured from an ovarian cortical biopsy before OTC. The ovarian volume (ml) of entire ovaries excised for OTC was also monitored.

*Results:* There was no significant difference in mean age and in follicular density between group 1 and group 2. In contrast, ovarian volume and total number of ovarian cortex chips cryopreserved were significantly lower in patients who received gonadotoxic treatment before OTC (ovarian volume 5.3 ±3.1 ml vs 2.9 ±2.1 ml (mean ±SD), p=0.02), number of cortex chips: 21.3 ±8.1 vs 15.2 ±7.1, (mean ±SD), p=0.02). The reduction in the estimated ovarian reserve ranged from 10-20% in children to around 30% in adolescent girls (> 10 years).

*Conclusion:* Girls under the age of 10 tolerate a gonadotoxic insult better than adolescents, who may experience up to a 30% reduction in the ovarian reserve by first line gonadotoxic treatment, which today is considered having little effect on the follicle pool. This information will improve counselling of young female cancer patients, to decide for fertility preservation or not.

**Key words:** ovarian tissue cryopreservation, fertility preservation, ovarian volume, follicle density.

**Introduction**

During the last 50 years, children and adolescents diagnosed with cancer have progressed from having a serious potentially fatal disease to having a most often curable disease. The five-year survival for all cancer types is estimated to be more than 80% in both children and adolescents (1). Furthermore, the late-effect mortality from any cause has significantly decreased across the last decades among five-year survivors of childhood cancer according to the Childhood Cancer Survivor Study (2). This success comes from a remarkable development of effective therapeutic regimens, including alkylating agent-based chemotherapy and radiotherapy.

This progress has created an awareness of quality of life aspects after cancer highlighting that the successful treatment may compromise fertility after recovery (3). The pool of ovarian follicles that constitute the reproductive potential of a girl may be severely reduced or disappear as a consequence of the treatment required (4). In prepubertal girls the only available option to preserve fertility is to cryopreserve ovarian tissue (5, 6). If ovarian activity is destroyed and premature ovarian insufficiency (POI) occurs in young female survivors, cortical ovarian tissue might be transplanted to restore ovarian function either for fertility purposes (7, 8) Recently, the first child being born after transplantation of ovarian tissue that was harvested before menarche was reported (9).

However, it is often difficult to decide whether it is necessary for a young girl with a cancer disease to undergo an invasive procedure to obtain ovarian tissue for fertility preservation. The fertility preservation intervention is recommended when there is an estimated risk of POI exceeding 50% (10, 11). Ovaries from girls and young women contain very high numbers of follicles and may tolerate a relatively high gonadotoxic insult without losing all follicles (12). In most cases treatment is initiated with low risk regimens but if more aggressive treatment is needed, harvesting ovarian tissue for fertility preservation will be considered. The question therefore arises as to whether ovarian tissue cryopreservation (OTC) should be considered in connection with the first line treatment such as ABVD, which is often considered to cause a relatively mild gonadotoxic insult. Thus, what is the potential gonadotoxic insult caused by the first-line treatment on the fertility potential in prepubertal girls and adolescents?

To answer this question the present study evaluated the follicular density and ovarian volume in our cohort of young girls below the age of 18 years with respect to whether or not they had

received gonadotoxic treatment before OTC.

## Material and Methods

*Patients*

This retrospective study included a total of 63 girls younger than 18 years (range: 1.5 –17.9 years) diagnosed with a cancer and referred to one of the three centers that participate in the Danish program for fertility preservation by OTC, between year 2002 and 2014. The number of patients who had not received chemotherapy prior to oophorectomy was 31 (group 1), while a total of 32 patients (group 2) had received gonadotoxic treatment before ovarian excision. All patients in group 2 had been treated for an original oncological diagnosis, had experienced a relapse and underwent OTC prior to further treatment. Patients were only included if a biopsy of the ovarian cortex was spared for histology in connection with OTC.

*Procedure*

The ovarian cortex was isolated by manual dissection and cut into pieces of approximately 5 x 5 mm and 1 mm thickness and frozen by slow-freezing technique as previously described (13, 14). A small ovarian cortical biopsy (≈ 2x2x1 mm) is routinely taken for histological examination before freezing. The piece was processed for histology, cut into 30-µm sections, and stained with periodic-acid Schiff reagents and Mayer hematoxylin. The follicular density, follicles per $mm^3$, was calculated by counting all types of follicles in every second section as previously described (15). Since one entire ovary was removed, the ovarian volume was recorded by weighing the tissue prior to preparation for cryopreservation. The density of ovarian tissue has previously been determined to be 1 g/ml (16) using tissue weight and volume calculated by insertion in 0.9% NaCl solution. The ovarian surface area was calculated assuming that the ovarian volume represented a spherical structure.

*Statistics*

Microsoft Excel version 14.6.1 was used to analyse the data. The data for each variable for the pretreated and non-pretreated groups were symmetrically distributed with similar variances between group 1 and group 2, hence Student's t-tests assuming equal variance were performed to

compare between-group means of follicular density, ovarian volume and number of ovarian cortex pieces (17). Age-adjusted comparisons were not performed due to the similar age characteristics (mean, median, IQR, range) between two groups. P-values lower than 5% were considered statistically significant throughout the study. Quadratic regression curves were fitted to the data for both groups in order to visualise the similarities and differences reported (Figs 1 and 2), and in order to estimate the age-related loss in ovarian reserve following treatment (Table 2).

*Ethical approval*

The project of ovarian tissue cryopreservation was approved by the ethical Committee of Copenhagen and Frederiksberg (H-2-2001-044). The storage and collection of patient data was approved by the Ministry of Health (J.no 30-1372) and by the Danish Authorities to comply with EU-tissue directive.

**Results**

There was no significant difference in mean age (±SD) between group 1 and group 2 (13.2 ±4.1 vs 11.6 ±4.3 years; p=0.19, Supplementary Figure 1). Cancer diagnoses for patients in the two groups are listed in Table 1. In group 1, the most frequent diagnoses were Hodgkin's lymphoma (n=8) and Ewing sarcoma (n=6), while hematological malignancies (acute lymphoblastic leukemia and acute myeloid leukemia, n=14) were the most frequent in group 2. The chemotherapy regimens used in group 2 were all classified as having a low or moderate gonadotoxic impact as for instance low dose alkylating agents. However, it has not been possible to recover the actual cancer treatment administered prior to excision of ovarian tissue. In all 63 one-sided oophorectomy was performed to harvest ovarian tissue. No surgical complication was reported in connection with oophorectomy.

There was no significant difference in follicular density in the ovarian cortex between patients who received first-line chemotherapy prior to OTC and patients who did not ($p$>0.10)(Figure 1). In contrast, the ovarian volume was significantly higher in group 1 than in group 2 (mean ±SD 5.3 ±3.3 vs 2.9 ±2.1 ml, $p$<0.05) (Figure 2). Similarly, the total number of ovarian cortex chips cryopreserved was higher in patients who did not receive cancer treatment before ovary removal (mean ±SD 21.3 ±8.1 vs 15.2 ±7.1, $p<0.05$)(Supplementary Figure 2). The ovarian surface area was calculated assuming that the volume represents a spherical structure. The pool of primordial follicles is situated approximately one millimeter below the ovarian surface epithelium and the surface area therefore represents the ovarian pool. Further, this applies to both groups since the average estimated follicular density was similar between

the two groups. After cancer treatment the estimated ovarian surface area was reduced by around 10% in young girls and around 30% in adolescent girls (Table 2).

**Discussion**

The current study found no significant differences in terms of follicular density between young girls who received first-line chemotherapy before OTC and those who did not. On the other hand, the ovarian volume and the total number of ovarian cortex pieces were significantly reduced by cancer treatment received before OTC. Thus, the ovarian reserve and the future fertility potential, could – depending on age – be reduced by the first-line chemotherapy by almost 30% in girls 10—18 years of age. In this study patients who received low-risk treatment initially did not receive fertility preservation but were referred to OTC following relapse that required intensive cancer treatment with high gonadotoxicity justifying OTC. The cryopreservation process of freezing and transplantation of ovarian tissue is far from efficient – only a fraction of the transplanted follicles survive the entire process and becomes available to the patient. However, young girls lose only of average 10% of their pool, which is a low reduction and may be without long-term consequences for fertility. Some of the adolescent girls, who may lose around 30% of the pool of follicles, could in some cases be considered differently and would have benefitted from having had OTC performed initially, rather than after gonadotoxic treatment with only 70% of the original pool of follicles left, as this study suggests. However, the fact that relapse would occur was unforeseen in the first place. The present new information provides potential support for the patient and her parents. The information may qualify the difficult decision on whether to offer OTC to patients undergoing low-risk cancer treatments or only offering it to patients who are undergoing intensified chemotherapy.

We acknowledge that our study has limitations. The first-line treatments for group 2 are not homogeneous, although they are all estimated to be mildly or moderately gonadotoxic. All acute leukemia cases are in group 2, meaning that specific comparisons involving ALL and AML are not available from our data. The measurement of AMH in both groups would have had a positive contribution to our analyses and comparisons.

In light of the increased long-term survival of childhood cancer, counselling about fertility preservation is now recommended to be offered to young patients and their parents prior to any cancer treatment (18). The OTC is the only option available in girls and young women where

ovarian stimulation for oocyte/embryo cryopreservation is considered inappropriate. A substantial heterogeneity of inclusion criteria exists, not only worldwide, but also within single countries showing that the selection of patients for OTC is a new emerging area, where actual clinical experience is scarce especially considering the effect of transplantation. The procedure is usually offered when there is a high risk of POI (> 50%). However, in one of the largest cohorts of OTC in girls younger than 16 years, Jadoul and co-workers (19) demonstrated that it is difficult to estimate the risk of infertility, whereas Wallace and co-workers had success in predicting the gonadotoxic insult by a given cancer treatment (11, 20). It is possible to define treatment regimens as low-, medium- and high-risk for ovarian toxicity, but disease evolution is never totally predictable and currently an individual assessment is required in each individual case (19-21). An interesting avenue of future investigation based on similar data would be the comparison of ovarian characteristics following intensive chemotherapy for Ewing's (with ifosfamide) as opposed to treatment for ALL with much less exposure to alkylating agents.

Clinically the consequences of an up to 30% reduction of the ovarian reserve in young girls are not known. It is likely that the fertility potential is not significantly reduced in perhaps the first decades of life, but the risk of POI and infertility is related to the different treatment regimens. However, in order to preserve fertility later in life, oocytes or embryos can be cryostored when there still is a follicular reserve after recovery. Of course this requires additional treatment, which potentially could have been avoided.

Further, very little is known about the pre-pubertal ovary and the mechanisms occurring during the transition from childhood through puberty to adulthood. In the young ovary, the follicular density is higher (15, 22), and follicles in early stages of development are present both in the cortex and in the medulla (23, 24). A recent paper showed that ovarian tissue from pre-pubertal girls contains a large population of abnormal primordial follicles that are lost in adolescence (25). Moreover, immature follicles collected from pre-pubertal ovarian tissue may have a more limited capability of follicular development than those retrieved from adult tissue (25-28). Similarly, data about the ovarian volume and follicle distribution are very limited in young girls and adolescents. According to a normative model, the ovarian volume enlargement occurs through childhood and adolescence to reach the maximum volume at 20 years of age, thereafter declining towards the menopause and beyond (29); with respect to this model our non-treated volumes are close to the

predicted values and the treated volumes are substantially smaller. Antineoplasic drugs generally affect the growing follicles, but are also able to raise the recruitment of non-growing follicles and damage the ovarian vascularization in the stromal tissue with a detrimental effect on the ovarian reserve (30). These observations obviously make it even more difficult to evaluate the mechanisms of gonadotoxicity in young and adolescent girls and make the actual estimations of the gonadotoxic insult of the present paper important. Further, it is interesting to notice that the follicular density remains similar between the two groups (with densities for both groups close to predicted values from a normative model (31)), while the ovarian volume is reduced in the treated group. Perhaps the follicular density and overall three dimensional environment is important for determining initiation of follicular growth and recruitment of interstitial cells to theca cell layer (32).

Patients in group 2 who received cancer treatment prior to OTC in the majority of cases had a diagnosis of leukaemia. However, there are no leukemic patients in group 1 and it is not possible to directly evaluate the gonadotoxic insult by the pretreatment given to leukemic patients. However, the pretreatment given to leukemic patients is usually not considered to exert a strong gonadotoxic effect. This may be different with Ewing and other types of sarcoma, where pretreatment may include alkylating agents. However, there is no statistical significant difference between the follicular density of patients who did or did not receive pretreatment with this diagnosis. There is probably considerable inter individual variations and a larger data set is possibly required to unravel to potential differences in the gonadotoxic insult caused by specific regimes.

In conclusion, in girls under the age of 10 years first-line cancer treatment does not compromise the ovarian reserve with more than 10%. In contrast, adolescent girls between 11—18 years may experience an estimated reduction of 30% of their ovarian reserve. The precise long-term consequences of having a 30% reduced ovarian reserve are not known today but the information is important in the counseling of the young patients and their parents and to determine whether fertility preservation should be performed or not.

**Acknowledgement**

The Child Cancer Foundation in Denmark, The Novo Nordisk Foundation and the EU interregional project ReproHigh/ReproUnion are thanked for having funded this study. They had no role in the study design, collection and analysis of data, data interpretation or in writing the report.

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**Figure legends**

**Figure 1. Follicular density in ovarian cortex.**
There was no significant difference in follicular density in ovarian cortex from patients who did or did not receive first-line antineoplastic treatment. The lines are quadratic best-fit to the data for the two groups.

**Figure 2. Ovarian volume.**
The ovarian volume was significantly reduced by previous antineoplastic treatment as compared to those who did not receive pre-treatment ($p< 0.05$). The lines are quadratic best-fit to the data for the two groups.