Fertility and Sterility

Evaluation of ovarian tissue transplantation- results from three clinical centers

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Corresponding Author:	Dror Meirow, M.D. Chaim Sheba Medical Center at Tel Hashomer Tel Hashomer, ISRAEL
First Author:	Moran Shapira
Order of Authors:	Moran Shapira
	Marie-Madeleine Dolmans
	Sherman Silber
	dror meirow
Abstract:	Objective: To report ovarian tissue autotransplantation (AT) results and describe the relationship between technical/clinical factors and outcomes. Design: Multicenter retrospective cohort study Setting: Three tertiary medical centers. Patients: Infertile patients who had stored ovarian tissue before sterilizing treatment and returned for AT with the aim of conceiving. Interventions: Ovarian tissue cryopreservation (OTC) and AT, endocrine monitoring, in vitro fertilization Main outcome measures: Endocrine performance, pregnancy and live-birth rates . Results: Between 2004-2018, 70 patients underwent 87 ATs. Sixty patients undergoing 70 ATs met the inclusion criteria. Following AT, menses returned in 94% of patients and median follicle-stimulating hormone dropped from 68 to 19 IU/mL. Fifty pregnancies and 44 deliveries were obtained, with 50% of women achieving at least one pregnancy and 41.6% at least one delivery. Twelve patients underwent AT more than once and had their endocrine activity restored in case menses recurred after the first transplantation. Repeat transplantations yielded 5 live births in 3 patients, two of whom had already given birth following the first transplantation. Pre-harvesting chemotherapy wasn't associated with inferior outcomes. Of 7 patients whose pelvis was exposed to radiation before AT, 4 conceived and delivered. Neither tissue dimensions nor surgical approach affected fertility outcomes. Conclusion: OTC is highly effective at restoring fertility in sterilized patients and prior exposure to chemotherapy should not be considered a contraindication. Repeat AT should be contemplated in case of graft malfunction, especially if previous transplantation was successful. In selected cases, conception and delivery may be feasible after pelvic exposure to radiation

Full Title	Evaluation of ovarian tissue transplantation- results from three clinical centers
Authors	Shapira Moran[1] MD Dolmans Marie-Madeleine[2*] MD PhD Silber Sherman[3*] MD Meirow Dror[1*#] MD
Affiliation	1- Fertility Preservation Center, Division of Obstetrics and Gynecology, Sheba Medical Center, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel 2- Pôle de Gynécologie, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain, 1200 Brussels, Belgium; Gynecology Department, Cliniques Universitaires Saint Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium 3- Infertility Center of St. Louis, Saint Louis, Missouri , USA * similar in author order # Corresponding author
Corresponding Author	Dror Meirow meirow@tauex.tau.ac.il
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Abstract

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Design: Multicenter retrospective cohort study

Setting: Three tertiary medical centers.

Patients: Infertile patients who had stored ovarian tissue before sterilizing treatment and returned for AT with the aim of conceiving.

Interventions: Ovarian tissue cryopreservation (OTC) and AT, endocrine monitoring, in vitro fertilization

Main outcome measures: Endocrine performance, pregnancy and live-birth rates.

Results: Between 2004-2018, 70 patients underwent 87 ATs. Sixty patients undergoing 70 ATs met the inclusion criteria. Following AT, menses returned in 94% of patients and median follicle-stimulating hormone dropped from 68 to 19 IU/mL. Fifty pregnancies and 44 deliveries were obtained, with 50% of women achieving at least one pregnancy and 41.6% at least one delivery. Twelve patients underwent AT more than once and had their endocrine activity restored in case menses recurred after the first transplantation. Repeat transplantations yielded 5 live births in 3 patients, two of whom had already given birth following the first transplantation. Pre-harvesting chemotherapy wasn't associated with inferior outcomes. Of 7 patients whose pelvis was exposed to radiation before AT, 4 conceived and delivered. Neither tissue dimensions nor surgical approach affected fertility outcomes.

Conclusion: OTC is highly effective at restoring fertility in sterilized patients and prior exposure to chemotherapy should not be considered a contraindication. Repeat AT should be contemplated in case of graft malfunction, especially if previous transplantation was successful. In selected cases, conception and delivery may be feasible after pelvic exposure to radiation

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Introduction

In recent years, we have become increasingly aware of the long-term adverse effects of cytotoxic treatment, like ovarian failure and infertility, among young cancer survivors (1). This has prompted frequent inclusion of fertility preservation measures in the management of patients likely to overcome their disease, but at major risk of significant ovarian injury (2, 3). While storage of embryos and oocytes has become an accepted approach in the field, it is only recently that ovarian cortex cryopreservation has been adopted by a number of centers as a standard procedure for fertility preservation (4-6).

Ovarian tissue cryopreservation (OTC), which enables preservation of many thousands of primordial follicles at once (7), offers some major advantages over embryo and oocyte cryopreservation. It does not require ovarian stimulation and is the only option available for prepubertal girls and patients who cannot delay their cancer treatment to undergo stimulation (8). In terms of efficacy and success rates, according to some publications reporting cumulative data from several centers, about 30% of autotransplanted patients conceive and deliver with the aid of this procedure (9-11). However, the current literature indicates that despite being increasingly applied, OTC and autotransplantation (AT) are practiced quite differently by various teams worldwide. In particular, there is inconsistency regarding selection criteria for the procedure (age and chemotherapy exposure), laboratory handling of harvested tissue (fragment dimensions, freezing protocols), and the transplantation technique itself (transplantation site, surgical strategy). Thus, there is a growing need to better define selection criteria and determine the optimal technical approach.

This manuscript presents the combined results of OTC-AT from three different centers all with more than 15 years of experience in tissue transplantation. Each participating center independently coordinates the process of OTC and AT from beginning to end, meaning that patient selection, tissue harvesting, preparation, cryopreservation, storage and AT, and patient follow-up are performed exclusively on site. The current study takes into account the different strategies of each of the centers in order to provide

generalizable OTC-AT success rates and investigate the relationship between key technical/clinical factors and procedure outcomes.

Methods

Patients

This multicenter cohort study included three participating centers; the Sheba Medical Center (Israel), Cliniques Universitaires Saint-Luc (Belgium) and the Infertility Center of St. Louis (USA). Included in this study were patients who underwent OTC for the purpose of fertility preservation prior to potentially sterilizing treatment and were auto-transplanted years later with the aim to conceive, while suffering from ovarian failure or after failing to conceive on IVF. Patients who were lost to follow up, who had undergone OTC due to other indications or whose tissue was cryopreserved using vitrification were excluded.

As of June 2018, a total of 1314 patients have undergone OTC in these three centers (545 in the Sheba medical center, 706 in Cliniques Universitaires Saint-Luc and 63 in the Infertility Center of St. Louis), Between January 2004 and June 2018, 70 women who had had their ovarian tissue stored came back to have it thawed and autotransplanted (34 from the Sheba medical center, 25 from the Cliniques Universitaires Saint-Luc,11 from the Infertility Center of St. Louis), leading to a combined return rate of 5.32%. Of the 70 returning patients, 10 were excluded due to the following reasons: Four had their tissue cryopreserved using vitrification (Infertility Center of St. Louis), two were lost to follow up, 2 were harvested due to premature ovarian insufficiency one had borderline tumor and an additional patients was found to be 6 weeks pregnant a month following transplantation. Thus, a total of 60 transplanted patients who met the inclusion criteria were finally included in the study.

Patient age at the time of OTC was between 14 and 39 years (mean 26.3 ± 6.26) and the majority of subjects were nulliparous (83.05%). Most of them were diagnosed with hematologic malignancies (23 Hodgkin's lymphoma, 11 non-Hodgkin's lymphoma, 5 leukemia). Five patients had breast cancer, 2 cervical cancer, 2 central nervous system (CNS) tumors, 2 sarcomas, and one colorectal cancer. The remaining 9 patients underwent OTC for a variety of non-malignant conditions that required potentially sterilizing medical

treatment, including endometriosis resection surgery involving bilateral salpingo-oophorectomy, bone marrow transplantation for sickle cell disease, and chemotherapy including alkylating agents for systemic lupus erythematosus, scleroderma and Wegener's granulomatosis. Twenty-four patients were treated with non-sterilizing chemotherapy prior to tissue harvesting; 10 patients had tissue harvested upon initial diagnosis after non-sterilizing chemotherapy but before more intensive chemotherapy, and 13 patients at the time of a later relapse that required second-line therapy. In one sarcoma patient, tissue was harvested during surgery to remove a large abdominal tumor after several chemotherapy cycles. Oncologic treatment included pelvic exposure to radiation in 7 patients; two cervical cancer patients and one colorectal cancer patient were given pelvic radiotherapy, two leukemia patients and one Hodgkin's lymphoma patient were treated with total body irradiation, and one non-Hodgkin's lymphoma patient underwent lumbo-aortic radiation.

At the time of AT, a median of 7 years after OTC (interquartile range [IQR] 3.8-10.2), mean patient age was 32.7 ± 5.6 years (range 21-45 years). Most patients showed clear evidence of ovarian failure, with persistent amenorrhea and hormone profiles indicative of menopause, and median follicle-stimulating hormone (FSH) levels of 63.8 IU/mL (IQR 38.5-90.3). However, 8 patients did show some signs of residual ovarian activity, with 7 experiencing regular menses and one oligomenorrhea. Transplantation was performed in these cases in order to treat their infertility and enhance their chances of future pregnancy. Five of these patients manifested low (<0.1ng/mL) anti-Müllerian hormone (AMH) and/or high (>15 IU/mL) FSH levels. Each underwent several in vitro fertilization (IVF) cycles, all of which resulted in up to one embryo transfer per patient, but no pregnancies were achieved. Two 45-year-old patients underwent transplantation due to age-related poor reproductive performance, with one suffering repeated implantation failure, and the other, multiple early miscarriages. Finally, the colorectal cancer patient had one ovary harvested and the other transposed to the upper abdomen. Laparoscopy was subsequently performed to replace the right ovary inside the pelvis, and this opportunity was taken to transplant ovarian tissue

fragments to boost the chances of pregnancy by IVF. Institutional review board approval was obtained for the study.

Ovarian tissue cryopreservation and transplantation

For a detailed description of laboratory and surgical methods, readers are referred to previous publications (4, 12-14). In brief, following tissue extraction, the tissue was cut into fragments, measuring 1-2 mm in thickness. The median surface area of these fragments was 50 mm^2, ranging from 2 mm^2 to 60 mm^2 (table 1). Slow-freezing was applied in all cases, using the protocol described by Gosden and colleagues with minor modifications (15). Tissue harvesting, preparation, OTC and storage were done on site in all centers, with no transportation needed.

Most patients included in the study underwent AT only once. However, in several women, repeat transplantation was considered in case of graft malfunction, as indicated by persistently high FSH levels and cessation of menses, or no resumption of menses in the first place. Thus, 12 patients were grafted more than once, the number of transplantations totaling 76 for all 60 patients (Table 1,3).

Orthotopic reimplantation was performed in all cases, using the minilaparotomy approach in 42 transplantations and the laparoscopic approach in 34 transplantations. The median number of strips grafted on each occasion was 5.5 (IQR 4-13). Small ($\leq$9 mm^2) tissue strips were transplanted in 10 patients, medium (10-49 mm^2) tissue strips in 9 patients, and large ($\geq$ 50mm^2) tissue strips in 37 patients. One patient was grafted with both medium and large strips. In 3 additional subjects, tissue dimensions were not recorded. The fragments were grafted to the ovary inside subcortical ovarian pockets (16) or onto a denuded ovarian medulla (12, 14). Another site used was a specially created peritoneal window in the lower pelvis (Table 1).

Following AT, ovarian function was closely monitored on site, including keeping track of the first menstrual bleed and repeated day 3 FSH measurements upon return of menses. The three participating centers differed from one another with respect to clinical management after confirmation of graft activity. In Israel, assisted

reproductive technology (ART) is relatively accessible and IVF treatments are generally funded by national authorities. Therefore, in the Sheba Medical Center, after resumption of menses, increase in estradiol (E2) levels and demonstration of follicle growth by ultrasound, proceeding to IVF was promptly considered. IVF was performed using either modified natural cycles (17) or the standard gonadotropin-releasing hormone (GnRH) antagonist protocol, as dictated by individual endocrine profiles and antral follicle counts. By contrast, at the Infertility Center of St. Louis (USA), patients were encouraged to conceive spontaneously, without any intervention such as IVF or hormonal stimulation. At Cliniques Universitaires Saint-Luc (Belgium), IVF was not routinely performed after transplantation. It was proposed in the presence of male factor or mechanical infertility, or when spontaneous pregnancy had not occurred within one year of AT. The protocol involved mild stimulation, followed by GnRH antagonist administration once a leading follicle measuring >10 mm was detected (18).

Statistical analysis

Statistical analysis was conducted using SciPy, version 1.0.0. Normality of distribution was evaluated by histograms and Q-Q plots. Continuous variables were presented as means and standard deviation (SD) or medians and IQR, as appropriate. Categorical variables were presented as numbers and percentages. Normally distributed continuous variables were compared using Student's t-test, and non-normally distributed numbers were compared using the Mann-Whitney test. Categorical variables were compared using the chi-squared or Fisher's exact test, as appropriate. A p-value <0.05 was considered statistically significant.

Results

During the study period, 60 patients that met the inclusion criteria underwent 76 ATs. After the first transplantation, there was evidence of ovarian activity in 55 women. Of the 52 patients who showed no ovarian function prior to AT, 47 (90.38%) resumed menses on average of 3.64 ± 1.79 months following transplantation. Among the five women who did not recover menses, one had undergone hysterectomy, and

another was treated with chemotherapy after being diagnosed with secondary malignancy shortly after transplantation. Therefore, excluding these two patients, the restoration rate of menses was 94% (47/50). Median FSH levels before AT were 63.8 IU/mL (IQR 38.5-90.3), and after AT, 19 IU/mL (IQR 10.7-36.5).

After transplantation, the patients were followed for a median duration of 64 months (IQR 36-82). During this time period, 33 of them underwent ovarian stimulation and IVF, 14 (42.4%) conceived at least once. A total of 117 cycles were completed, with a median three cycles per patient (IQR 2-5). In 25 of these cycles (21.36%), attempts to retrieve oocytes revealed empty follicles. One hundred and sixty-nine oocytes were collected and 88 were fertilized, yielding a fertilization rate of 52.07%. Altogether, 71 embryos were transferred. Mean oocyte number per cycle was 1.44, with clinical pregnancy and live birth rates per cycle of 14.5% and 12.8% respectively. Ultimately. A total of 17 pregnancies and 15 live births were obtained following IVF.

A total of 50 pregnancies were achieved (33 spontaneous, 17 IVF), 44 of which resulted in live births. Three of the pregnancies are still ongoing. Six patients conceived and delivered more than once; Four patients gave birth twice and 2 patients had 3 deliveries (one of them conceived 4 times and had spontaneous miscarriage). The overall miscarriage rate was 6%: 11.76% for IVF pregnancies and 3.03% for spontaneous pregnancies (p=0.29). Thirty patients conceived at least once (50%) and 25 gave birth at least once (41.6%). Two additional pregnancies were obtained in 2 out of 4 patients transplanted with vitrified tissue at the Infertility Center of St louis (12) but, as previously stated, these women were excluded from the analysis. Patients who conceived were compared to those who did not. Mean age on OTC was 25±5.5 vs. 27.6±6.8, respectively, but this difference did not reach statistical significance (p=0.1). However, there was a trend towards a higher proportion of women aged ≥35 years on OTC among those who did not conceive (53.3% vs 26.6%, p=0.06). In addition, a significantly younger age on AT was observed among those who became pregnant (31.6±5.2 vs 34.8±6.2, p=0.03). Hematologic malignancy was the most common diagnosis in both groups (70% vs 60%).

Among patients grafted with small tissue fragments, 58% conceived at least once as opposed to 46% of those transplanted with medium/large tissue fragments, but the difference was not statistically significant (p=0.41). The surgical approach did not impact outcomes; of those transplanted using a laparoscopic approach, 44.73% conceived at least once, compared to 55.26% of those undergoing AT by minilaparotomy (p=0.7). Of those whose tissue had been transplanted into ovarian site 51.5% conceived at least once, compared to 50% of those whose tissue had been transplanted into peritoneal pockets (=0.71).

Twenty-four women had their tissue stored after chemotherapy. Patients affected by hematologic malignancies were most likely to be exposed to chemotherapy before OTC (Figure 1). Table 2 shows transplantation outcomes according to chemotherapy treatment prior to OTC. Those who received and did not receive chemotherapy were of similar age. There was no significant difference between the two groups in terms of post-transplantation FSH values, restoration of menses, pregnancy, live birth, or miscarriage rates. Ten patients were treated with alkylating agents before OTC and exhibited no ovarian activity before AT. After AT, seven had their menses restored and four conceived at least once. The four patients who conceived were aged 23-32 years at OTC. One non-Hodgkin's lymphoma patient had been treated with 6 cycles of VACOP-B (etoposide, doxorubicin, cyclophosphamide, vincristine, prednisolone, bleomycin) before OTC and conceived via IVF following transplantation. The second, a Hodgkin's lymphoma patient, had been given 5 cycles of ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) and one cycle of MOPP (mechlorethamine, vincristine, procarbazine, prednisone) and conceived spontaneously after AT. The third, another Hodgkin's lymphoma patient, had undergone 6 cycles of BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone), conceived after two IVF attempts, and is currently 8 weeks pregnant. Finally, a breast cancer patient who received 4 cycles of AC (adriamycin and cyclophosphamide) and a further two cycles of cyclophosphamide, conceived after transplantation following just one IVF attempt.

Seven patients had their pelvis exposed to radiation prior to AT. All four leukemia/lymphoma survivors who underwent total body or lumbo-aortic radiation, and received pelvic radiation of up to 20Gy, regained

their menses following transplantation. Three of them conceived and delivered at term, with no obstetric complications, demonstrating preserved uterine function despite exposure to radiation. One colorectal cancer patient who was given pelvic radiation at a total dose of 45Gy also conceived after AT and replacement of a previously transposed ovary back to the pelvis. She delivered prematurely at 32 weeks of gestational age. By contrast, the two cervical cancer patients did not recover endocrine function and did not achieve pregnancy.

Twelve patients underwent more than one transplantation (Table 3); four of them underwent 3 ATs, and the remaining 8, 2 ATs. Mean age at OTC was 24.17 ± 4.62, while at first and last AT, it was 29.92 ± 3.84 and 32.83 ± 3.66 respectively. The median interval between transplantations was two years (range 1-5). All those who had their menses restored after the first transplantation also recovered menses following subsequent transplantations. In three patients, repeated ATs resulted in a total of 5 pregnancies and live births. Two of these women had also conceived following their first transplantation.

Discussion

The years 2004-2005 saw some groundbreaking advances in the world of reproduction. In 2004, the first live birth to be achieved spontaneously following OTC-AT was reported in Belgium (14). In 2005, the first live birth resulting from ovarian stimulation and IVF following OTC-AT in a woman with documented long-standing ovarian failure was announced in Israel (16). During that same year, a live birth obtained after transplantation of fresh ovarian cortex from a monozygotic twin was also recorded for the first time in the USA (19). In the 15 years that have passed since those transformative times, OTC-AT has gained ground as an accepted means of fertility preservation, practiced in multiple centers around the world on both an experimental and non-experimental basis. As of June 2017, it was estimated that OTC-AT had yielded more than 130 live births worldwide (20). This figure is expected to swell over the coming years, as the appeal of OTC-AT grows by virtue of its low surgical complication rates (21) and favorable outcomes. In a meta-analysis, OTC-AT was found to restore endocrine function in 64% of cases, with an ongoing pregnancy/live birth rate of 28.4% (10). Similar live birth rates are often observed when

scrutinizing data from other teams or networks, for example 32.6% in the Spanish group in 2018 (22), 31% in the Danish group in 2015 (9) and 25% in the German FertiPROTEKT network in 2016 (11).

Our first three reports of successful ovarian tissue transplantation from 2004-2005 triggered a learning curve that continues to this day, and the current paper presents the actual clinical findings of this ongoing process. Restoration of ovarian activity was observed in 94% of patients who had shown no ovarian activity prior to AT. At least one pregnancy was obtained in 50% of patients, and 41.6% of subjects delivered at least once. Such encouraging figures support use of OTC when fertility preservation is desired, emphasize its role in restoration of endocrine function and, importantly, advocate implementation of OTC as a non-experimental procedure. The positive results obtained in the current multicenter study may be attributed to extensive long-term experience in OTC-AT, as well as complete centralization of the entire process by each of the centers involved. When compared to other series in literature, a younger study population may account for the higher pregnancy rate observed in our study; the current study included a large number of hematologic patients who were at their 20's on presentation. In contrast, other series on literature included a higher proportion of breast cancer patients, who underwent OTC at an older age (9, 11, 22). Substantiates this explanation is a trend towards a higher proportion of patients aged ≥35 on OTC among those who did not conceive in our cohort. Age differences could also explain the low miscarriage rate observed in this study (6%), as opposed to the one described by other groups (~33%) (9, 22)..

It is still unclear whether OTC is preferable to oocyte cryopreservation in postpubertal patients who have sufficient time for ovarian stimulation before chemotherapy. A study comparing the two techniques reported a trend towards higher pregnancy (40.8% vs 32.6%) and live birth (27.3% vs 18.2%) rates in patients undergoing oocyte vitrification (22). However, it is noteworthy that the favorable outcomes of oocyte vitrification presented in the study above are from fertility centers with vast experience in oocyte freezing. On the other hand, as previously stated, a number of groups around the world have achieved live birth rates of more than 18% following OTC-AT, and the results of the current analysis considerably outperform this figure. Moreover, OTC has several advantages over oocyte vitrification. It restores ovarian

function and enables natural conception, as reflected by the 33 spontaneous pregnancies achieved in this series (leading to a spontaneous clinical pregnancy rate of 55%). Another advantage demonstrated by the current cohort is that OCT-AT may result in several pregnancies. These are less likely to occur following a single cycle of IVF for oocyte cryopreservation. When possible, a combination of OTC and oocyte cryopreservation can be offered to further increase the chances of future pregnancy (23).

Importantly, ovarian stimulation and oocyte retrieval become irrelevant immediately after chemotherapy, and for at least another 6 months (8), resulting in a diminished response to ovarian stimulation (24, 25) and genetically abnormal oocytes, as demonstrated in animal models (26-28). Hence, OTC is the only fertility preservation option available to patients who cannot postpone chemotherapy upon primary diagnosis or disease relapse. There is still ongoing debate around the efficacy and feasibility of OTC in the context of previous chemotherapy, with some centers restricting the procedure to chemotherapy-naive patients only (29). However, in the present cohort, both chemotherapy-naive and chemotherapy-exposed patients showed similar ovarian activity restoration rates of about 94%. The proportion of women who conceived and delivered at least once was actually slightly higher among chemotherapy-exposed patients (58% vs 42% and 46% vs 39% respectively) despite being of similar age, but this difference was not statistically significant. These results corroborate those observed in a smaller recent study reporting outcomes of 31 autotransplanted patients (30). By one year after AT, ovarian function was restored in 93% of chemotherapy-exposed subjects as opposed to 67% of non-exposed subjects ($p=0.14$). None of the chemotherapy-naive patients conceived, while 32% of the chemotherapy-exposed group delivered at least once, although lower pregnancy rates were encountered among patients treated with alkylating agents. In our study, however, 7 out of 10 subjects previously exposed to alkylating agents regained ovarian function following AT. Four of them conceived and gave birth, despite exposure to cyclophosphamide. Due to a lack of statistical power, the findings of the present study cannot refute a possible decline in AT outcomes in case of preharvesting chemotherapy exposure, especially when alkylating agents are involved.

Nevertheless, chemotherapy-exposed patients did not show inferior results to chemotherapy-naive patients . Withholding OTC-AT from chemotherapy-exposed patients therefore seems unjustified.

The value of AT in patients with previous exposure to pelvic radiation is unclear. Assuming orthotopic transplantation is performed, poor pelvic vascularization may compromise graft function, making the procedure somewhat ineffective. Importantly, even if ovarian function is preserved or restored, radiation-induced uterine injury may impede uterine capability to carry a pregnancy, so surrogacy is often required. Our data suggest that AT may be appropriate in patients whose pelvis is exposed to radiotherapy, as four out of 5 patients receiving pelvic radiation at doses ≤ 45 Gy conceived and delivered. While patients who received radiation at doses ≤ 20 Gy delivered on term without any complications, the patient who was treated with a higher dose of 45 Gy delivered prematurely at 32 weeks. A higher dose of pelvic radiation (external and brachytherapy) is given in cases of cervical cancer (31), and none of the cervical patients in our cohort managed to conceive. to the best of our knowledge, there have been no reports of successful transplantation in such cases. When contemplating fertility preservation in a patient destined to undergo pelvic radiotherapy, informed discussion is essential. Limited experience with the procedure in such circumstances should be communicated to the patient, as should the possibility of alternative options and potential need for surrogacy in the future. A higher risk for numerous obstetric complications should be conveyed to the patient as well, including miscarriage, preterm labor and intra-uterine growth restriction (32). If AT is pursued, tissue should be grafted to a vascularized site, such as an ovary replaced back inside the pelvis after transposition.

Graft dimensions have been shown to play a significant role in determining the extent of follicle loss during cryopreservation, thawing and transplantation. Upon freezing, thinner cortical strips allow good penetration of cryoprotective agents and decrease the likelihood of ice crystal formation and injury (33). Upon transplantation, smaller dimensions reduce penetration depth for new blood vessels, potentially shortening the ischemic period (34, 35). On the other hand, grafting smaller fragments may boost follicle activation, resulting in increased loss of dormant follicles (36). The present study has enabled us to describe AT clinical

results according to fragment dimensions for the first time. Patients transplanted with small tissue strips ($\leq 9mm^2$) achieved similar pregnancy and live birth rates to those transplanted with larger pieces. Tissue dimensions may determine graft's life span, but such hypothesis could not be evaluated in the current study. In order to determine optimal tissue strip dimensions, if they exist at all, larger multicenter studies, also evaluating grafts' life span, should be conducted. As experience with OTC and transplantation continues to grow globally, such studies will undoubtedly be available in the near future.

In the event of graft malfunction, repeat AT can be considered if a sufficient amount of ovarian tissue was stored. To prevent complications of repeated surgical procedures, it is important to identify patients most likely to benefit from additional transplantation. In the present study, 12 patients underwent more than one AT. All those whose menses resumed following their first transplantation also exhibited ovarian activity after their subsequent transplantation. By contrast, the only woman not to recover her menses following her first transplantation did not show any ovarian activity after her second transplantation either. Repeat ATs yielded a total of 5 live births in 3 patients, two of whom had been transplanted for the second or third time after already giving birth following their first AT. These patterns may suggest that first and repeat transplantations share a similar course and outcomes. Hence, patients who manage to initially conceive following AT, but later experience diminished/absent graft activity, may be encouraged to pursue a second transplantation if an additional pregnancy is desired.

Several strength and limitations of the study should be acknowledged. Among the strengths of the current study is the centralization practiced by the three participating centers, exclusively managing every case through all aspects of patient care, from OTC to AT and subsequent follow up. Additionally, to the best of our knowledge, our study presents the largest series to evaluate the effect of chemotherapy exposure on AT outcomes, and is the first to describe pregnancy rates according to different surgical strategies. Yet, due to the limited years of OTC-AT practice combined with a low return rate of 5.3%, and despite the integration of data coming from three centers, the modest number of patients who underwent AT could limit the sensitivity of statistical tests. The effect size observed when comparing technical strategies was small,

limiting the statistical power to detect differences in pregnancy rates. The return rate in the current cohort is rather low, but nevertheless comparable to utilization rates observed by other groups, in oocyte vitrification(22), OTC-AT(11, 22) and male fertility preservation programs (37, 38). Improved identification of those likely to utilize their tissue is of great importance and should be addressed in future studies.

Conclusion

This multicenter study reinforces the role of OTC as an effective tool for fertility preservation, resulting in conception and live birth in up to 50% and 41.6% of patients respectively. Our study reveals that once a previously functioning graft is depleted, repeat AT is likely to trigger endocrine activity and conception once again. OTC is highly appropriate in case of recent chemotherapy and should not be excluded in such scenarios. AT following pelvic exposure to radiation can yield satisfactory results in selected cases, not always requiring surrogacy. However, the efficacy of AT remains questionable in case of cervical cancer. In terms of techniques used when handling harvested tissue, neither tissue dimensions nor the surgical approach to transplantation appear to affect reproductive outcomes, but these should be further evaluated in larger studies in the future. It is expected that in the coming years, implementation of OTC-AT will continue to expand globally, with increasing numbers of procedures performed for non-oncologic indications too. We can therefore expect a growing body of data to accumulate in the near future, allowing for large-scale studies to better understand and address the impact of technical aspects and selection criteria on outcomes, further improving efficiency and performance. In the meantime, it is fair to say that in the hands of experienced practitioners, OTC-AT is a highly effective approach to fertility preservation.

Table 1- Surgical methods practiced by each participating center

	Sheba Medical center	Cliniques Universitaires Saint-Luc	Infertility Center of St. Louis
Number of transplanted patients (number of ATs)	34 (39)	25 (34)	11(14)
Number of eligible patients[1] (number of ATs)	32(37)	23(32)	5(7)
Tissue Fragments dimensions (median)	$50mm^2$ (IQR 50-50, range 2-60)	19.5 mm^2 (IQR 7-45, range 4-64)	$50mm^2$ (IQR 50-50, range 36-50)
Number of fragments transplanted (median)	4 (IQR 3.8-6, range 2-20)	13 (IQR 5.1-22, range 3-70)	5 (IQR 2.8-7.5, range 2-9)
AT surgical approach	Minilaparotomy - 32/37 Laparoscopy - 5/37	Minilaparotomy - 3/32 Laparoscopy - 29/32	Minilaparotomy -7/7
Graft site	Ovary-subcortical pockets- 22/37 Peritoneal window- 1/37 Both -14/37	Ovary-denuded medulla- 18/32 Peritoneal window- 5/32 Both- 9/32	Ovary-denuded medulla -7/7

AT: autotransplantation
IQR: interquartile range
1- Patients whose tissue was vitrified and those who were harvested due to premature ovarian insufficiency were not included. See additional exclusion criteria on text.

Table 2 – Ovarian transplantation outcomes according to pre-harvesting chemotherapy exposure

	Total	Chemotherapy	No chemotherapy	p-value
Patients	60	24	36	
Age at OTC (years)	26.3 ± 6.26	25.83 ± 5.65	26.55 ± 6.71	0.66
Age at AT (years)	32.7 ± 5.6	32.96 ± 6.1	33.36 ± 5.64	0.80
Tissue transplanted (area, mm^2)	250 (150-300)	200 (150-214)	200 (165-310)	0.11
FSH after AT (IU/mL)	19 (10.7-36.5)	14 (8.8-42.5)	23.65 (12-34.2)	0.26
Menses recurrence[1]	(47/50) 94%	(19/20) 95%	(28/30) 93.3%	0.81
At least one pregnancy	(30/60) 50%	(14/24) 58.33%	(16/36) 44.4%	0.32
At least one live birth	(25/60) 41.66%	(11/24) 45.83%	(14/36) 38.9%	0.79
Number of pregnancies (IVF)	50 (17)	25 (8)	25 (9)	
Number of live births (IVF)	45 (15)	21 (5)	24 (10)	
Number of ongoing pregnancies (IVF)	3 (1)	1 (1)	2 (0)	
Miscarriages	(3/50) 60%	(3/25) 12%	(0/25) 0%	0.24

1- Ten patients were excluded from the analysis: eight patients who had residual ovarian activity prior to transplantation (N=8), one patient who underwent hysterectomy, and an additional patient who was treated with chemotherapy after being diagnosed with secondary malignancy shortly after transplantation

OTC: ovarian tissue cryopreservation
AT: autotransplantation
FSH: follicle-stimulating hormone
IVF: in vitro fertilization

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Table 3- Repeat ovarian tissue transplantations- patients characteristics and results

			OTC			Before AT			After AT			
Patient #	AT#	Diagnosis	Age	Chemotherapy regimen	Radiation	Age	Menses	FSH (IU/mL)	Menses	Time to menses	FSH (IU/mL)	Pregnancies/ live births
1	1	CML	21	/	TBI	27	no	116	yes	3M	7.2	
	2					28	no	44	yes	3M	100	
	3					33	no	50	yes	3M	14	
2	1	Ewing sarcoma	24	/	/	28	no	107	yes	3M	24.3	1/1 (IVF)
	2					31	no	80	yes	2M	9	1/1 (IVF)
3	1	Hodgkin's lymphoma	21	ABVD	/	28	no	71	yes	8M	21.5	
	2					29	no	35	yes	0.5M	31	
4	1	Non-Hodgkin's lymphoma	36	RCHOP	/	40	no	87	no	/	90	
	2					41	no	90	no	/	78	
5	1	Hodgkin's lymphoma	25	/	/	30	no	91	yes	4M	34	
	2					31	yes	34	yes	4M	NA	1/1 (spontaneous)
6	1	Sickle cell disease	20	/	/	26	no	44	yes	5M	22	
	2					27	yes	25	yes	4M	NA	
	3					31	yes	NA	yes	4M	25	
7	1	Non-Hodgkin's lymphoma	28	ABVD	/	34	no	65	yes	6M	80	
	2					36	yes	80	yes	2M	78	
8	1	Wegener's granulomatosis	22	/	/	27	no	114	yes	5M	60	
	2					28	yes	60	yes	4M	IVF	
9	1	Hodgkin's lymphoma	23	ABVD	/	30	no	72	yes	6M	103	
	2					33	yes	103	yes	5M	64	
	3					35	yes	64	yes	2M	NA	
10	1	Hodgkin's lymphoma	22	MOPP/ ABVD	/	28	yes	62	yes	6M	118	
	2					30	yes	41	yes	NA	NA	
11	1	Hodgkin's lymphoma	28	/	/	30	no	50	yes	9M	48	
	2					33	no	48	yes	6M	35	
12	1	Hodgkin lymphoma	20	ABVD	TBI	31	no	64.2	yes	4M	8.2	1/1 (Spontaneous)
	2					33	no	62.4	yes	4M	3.9	
	3					36	no	61.2	yes	4M	5	3/3 (Spontaneous)

OTC: ovarian tissue cryopreservation
AT: autotransplantation
FSH: follicle-stimulating hormone
CML: chronic myeloid leukemia
ABVD: doxorubicin, bleomycin, vinblastine, dacarbazine
MOPP: mechlorethamine, vincristine, procarbazine, prednisone)
RCHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone
TBI: Total body irradiation

Figure legends

Figure 1. Chemotherapy exposure according to diagnosis. Hematologic malignancies were more commonly associated with chemotherapy before ovarian tissue cryopreservation.

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Full Title	Evaluation of ovarian tissue transplantation- results from three clinical centers
Authors	Shapira Moran[1] MD Dolmans Marie-Madeleine[2*] MD PhD Silber Sherman[3*] MD Meirow Dror[1*#] MD
Affiliation	1- Fertility Preservation Center, Division of Obstetrics and Gynecology, Sheba Medical Center, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel 2- Pôle de Gynécologie, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain, 1200 Brussels, Belgium; Gynecology Department, Cliniques Universitaires Saint Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium 3- Infertility Center of St. Louis, Saint Louis, Missouri , USA * similar in author order # Corresponding author
Corresponding Author	Dror Meirow meirow@tauex.tau.ac.il
Running title	Ovarian auto-transplantation outcomes
Capsule	Multicenter study reporting a 50% pregnancy rate and 42% live birth rate following ovarian tissue transplantation
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2

Abstract

Objective: To report ovarian tissue autotransplantation (AT) results and describe the relationship between technical/clinical factors and outcomes.

Design: Multicenter retrospective cohort study

Setting: Three tertiary medical centers.

Patients: Infertile patients who had stored ovarian tissue before sterilizing treatment and returned for AT with the aim of conceiving.

Interventions: Ovarian tissue cryopreservation (OTC) and AT, endocrine monitoring, in vitro fertilization

Main outcome measures: Endocrine performance, pregnancy and live-birth rates.

Results: Between 2004-2018, 70 patients underwent 87 ATs. Sixty patients undergoing 70 ATs met the inclusion criteria. Following AT, menses returned in 94% of patients and median follicle-stimulating hormone dropped from 68 to 19 IU/mL. Fifty pregnancies and 44 deliveries were obtained, with 50% of women achieving at least one pregnancy and 41.6% at least one delivery. Twelve patients underwent AT more than once and had their endocrine activity restored in case menses recurred after the first transplantation. Repeat transplantations yielded 5 live births in 3 patients, two of whom had already given birth following the first transplantation. Pre-harvesting chemotherapy wasn't associated with inferior outcomes. Of 7 patients whose pelvis was exposed to radiation before AT, 4 conceived and delivered. Neither tissue dimensions nor surgical approach affected fertility outcomes.

Conclusion: OTC is highly effective at restoring fertility in sterilized patients and prior exposure to chemotherapy should not be considered a contraindication. Repeat AT should be contemplated in case of graft malfunction, especially if previous transplantation was successful. In selected cases, conception and delivery may be feasible after pelvic exposure to radiation

Key words: ovarian auto-transplantation; ovarian cryopreservation; fertility preservation; cancer;

Introduction

In recent years, we have become increasingly aware of the long-term adverse effects of cytotoxic treatment, like ovarian failure and infertility, among young cancer survivors (1). This has prompted frequent inclusion of fertility preservation measures in the management of patients likely to overcome their disease, but at major risk of significant ovarian injury (2, 3). While storage of embryos and oocytes has become an accepted approach in the field, it is only recently that ovarian cortex cryopreservation has been adopted by a number of centers as a standard procedure for fertility preservation (4-6).

Ovarian tissue cryopreservation (OTC), which enables preservation of many thousands of primordial follicles at once (7), offers some major advantages over embryo and oocyte cryopreservation. It does not require ovarian stimulation and is the only option available for prepubertal girls and patients who cannot delay their cancer treatment to undergo stimulation (8). In terms of efficacy and success rates, according to some publications reporting cumulative data from several centers, about 30% of autotransplanted patients conceive and deliver with the aid of this procedure (9-11). However, the current literature indicates that despite being increasingly applied, OTC and autotransplantation (AT) are practiced quite differently by various teams worldwide. In particular, there is inconsistency regarding selection criteria for the procedure (age and chemotherapy exposure), laboratory handling of harvested tissue (fragment dimensions, freezing protocols), and the transplantation technique itself (transplantation site, surgical strategy). Thus, there is a growing need to better define selection criteria and determine the optimal technical approach.

This manuscript presents the combined results of OTC-AT from three different centers all with more than 15 years of experience in tissue transplantation. Each participating center independently coordinates the process of OTC and AT from beginning to end, meaning that patient selection, tissue harvesting, preparation, cryopreservation, storage and AT, and patient follow-up are performed exclusively on site. The current study takes into account the different strategies of each of the centers in order to provide

generalizable OTC-AT success rates and investigate the relationship between key technical/clinical factors and procedure outcomes.

Methods

Patients

This multicenter cohort study included three participating centers; the Sheba Medical Center (Israel), Cliniques Universitaires Saint-Luc (Belgium) and the Infertility Center of St. Louis (USA). Included in this study were patients who underwent OTC for the purpose of fertility preservation prior to potentially sterilizing treatment and were auto-transplanted years later with the aim to conceive, while suffering from ovarian failure or after failing to conceive on IVF. Patients who were lost to follow up, who had undergone OTC due to other indications or whose tissue was cryopreserved using vitrification were excluded.

As of June 2018, a total of 1314 patients have undergone OTC in these three centers (545 in the Sheba medical center, 706 in Cliniques Universitaires Saint-Luc and 63 in the Infertility Center of St. Louis), Between January 2004 and June 2018, 70 women who had had their ovarian tissue stored came back to have it thawed and autotransplanted (34 from the Sheba medical center, 25 from the Cliniques Universitaires Saint-Luc,11 from the Infertility Center of St. Louis), leading to a combined return rate of 5.32%. Of the 70 returning patients, 10 were excluded due to the following reasons: Four had their tissue cryopreserved using vitrification (Infertility Center of St. Louis), two were lost to follow up, 2 were harvested due to premature ovarian insufficiency one had borderline tumor and an additional patients was found to be 6 weeks pregnant a month following transplantation. Thus, a total of 60 transplanted patients who met the inclusion criteria were finally included in the study.

Patient age at the time of OTC was between 14 and 39 years (mean 26.3 ± 6.26) and the majority of subjects were nulliparous (83.05%). Most of them were diagnosed with hematologic malignancies (23 Hodgkin's lymphoma, 11 non-Hodgkin's lymphoma, 5 leukemia). Five patients had breast cancer, 2 cervical cancer, 2 central nervous system (CNS) tumors, 2 sarcomas, and one colorectal cancer. The remaining 9 patients underwent OTC for a variety of non-malignant conditions that required potentially sterilizing medical

treatment, including endometriosis resection surgery involving bilateral salpingo-oophorectomy, bone marrow transplantation for sickle cell disease, and chemotherapy including alkylating agents for systemic lupus erythematosus, scleroderma and Wegener's granulomatosis. Twenty-four patients were treated with non-sterilizing chemotherapy prior to tissue harvesting; 10 patients had tissue harvested upon initial diagnosis after non-sterilizing chemotherapy but before more intensive chemotherapy, and 13 patients at the time of a later relapse that required second-line therapy. In one sarcoma patient, tissue was harvested during surgery to remove a large abdominal tumor after several chemotherapy cycles. Oncologic treatment included pelvic exposure to radiation in 7 patients; two cervical cancer patients and one colorectal cancer patient were given pelvic radiotherapy, two leukemia patients and one Hodgkin's lymphoma patient were treated with total body irradiation, and one non-Hodgkin's lymphoma patient underwent lumbo-aortic radiation.

At the time of AT, a median of 7 years after OTC (interquartile range [IQR] 3.8-10.2), mean patient age was 32.7 ± 5.6 years (range 21-45 years). Most patients showed clear evidence of ovarian failure, with persistent amenorrhea and hormone profiles indicative of menopause, and median follicle-stimulating hormone (FSH) levels of 63.8 IU/mL (IQR 38.5-90.3). However, 8 patients did show some signs of residual ovarian activity, with 7 experiencing regular menses and one oligomenorrhea. Transplantation was performed in these cases in order to treat their infertility and enhance their chances of future pregnancy. Five of these patients manifested low (<0.1ng/mL) anti-Müllerian hormone (AMH) and/or high (>15 IU/mL) FSH levels. Each underwent several in vitro fertilization (IVF) cycles, all of which resulted in up to one embryo transfer per patient, but no pregnancies were achieved. Two 45-year-old patients underwent transplantation due to age-related poor reproductive performance, with one suffering repeated implantation failure, and the other, multiple early miscarriages. Finally, the colorectal cancer patient had one ovary harvested and the other transposed to the upper abdomen. Laparoscopy was subsequently performed to replace the right ovary inside the pelvis, and this opportunity was taken to transplant ovarian tissue

fragments to boost the chances of pregnancy by IVF. Institutional review board approval was obtained for the study.

Ovarian tissue cryopreservation and transplantation

For a detailed description of laboratory and surgical methods, readers are referred to previous publications (4, 12-14). In brief, following tissue extraction, the tissue was cut into fragments, measuring 1-2 mm in thickness. The median surface area of these fragments was 50 mm^2, ranging from 2 mm^2 to 60 mm^2 (table 1). Slow-freezing was applied in all cases, using the protocol described by Gosden and colleagues with minor modifications (15). Tissue harvesting, preparation, OTC and storage were done on site in all centers, with no transportation needed.

Most patients included in the study underwent AT only once. However, in several women, repeat transplantation was considered in case of graft malfunction, as indicated by persistently high FSH levels and cessation of menses, or no resumption of menses in the first place. Thus, 12 patients were grafted more than once, the number of transplantations totaling 76 for all 60 patients (Table 1,3).

Orthotopic reimplantation was performed in all cases, using the minilaparotomy approach in 42 transplantations and the laparoscopic approach in 34 transplantations. The median number of strips grafted on each occasion was 5.5 (IQR 4-13). Small ($\leq$9 mm^2) tissue strips were transplanted in 10 patients, medium (10-49 mm^2) tissue strips in 9 patients, and large ($\geq$ 50mm^2) tissue strips in 37 patients. One patient was grafted with both medium and large strips. In 3 additional subjects, tissue dimensions were not recorded. The fragments were grafted to the ovary inside subcortical ovarian pockets (16) or onto a denuded ovarian medulla (12, 14). Another site used was a specially created peritoneal window in the lower pelvis (Table 1).

Following AT, ovarian function was closely monitored on site, including keeping track of the first menstrual bleed and repeated day 3 FSH measurements upon return of menses. The three participating centers differed from one another with respect to clinical management after confirmation of graft activity. In Israel, assisted

reproductive technology (ART) is relatively accessible and IVF treatments are generally funded by national authorities. Therefore, in the Sheba Medical Center, after resumption of menses, increase in estradiol (E2) levels and demonstration of follicle growth by ultrasound, proceeding to IVF was promptly considered. IVF was performed using either modified natural cycles (17) or the standard gonadotropin-releasing hormone (GnRH) antagonist protocol, as dictated by individual endocrine profiles and antral follicle counts. By contrast, at the Infertility Center of St. Louis (USA), patients were encouraged to conceive spontaneously, without any intervention such as IVF or hormonal stimulation. At Cliniques Universitaires Saint-Luc (Belgium), IVF was not routinely performed after transplantation. It was proposed in the presence of male factor or mechanical infertility, or when spontaneous pregnancy had not occurred within one year of AT. The protocol involved mild stimulation, followed by GnRH antagonist administration once a leading follicle measuring >10 mm was detected (18).

Statistical analysis

Statistical analysis was conducted using SciPy, version 1.0.0. Normality of distribution was evaluated by histograms and Q-Q plots. Continuous variables were presented as means and standard deviation (SD) or medians and IQR, as appropriate. Categorical variables were presented as numbers and percentages. Normally distributed continuous variables were compared using Student's t-test, and non-normally distributed numbers were compared using the Mann-Whitney test. Categorical variables were compared using the chi-squared or Fisher's exact test, as appropriate. A p-value <0.05 was considered statistically significant.

Results

During the study period, 60 patients that met the inclusion criteria underwent 76 ATs. After the first transplantation, there was evidence of ovarian activity in 55 women. Of the 52 patients who showed no ovarian function prior to AT, 47 (90.38%) resumed menses on average of 3.64 ± 1.79 months following transplantation. Among the five women who did not recover menses, one had undergone hysterectomy, and

another was treated with chemotherapy after being diagnosed with secondary malignancy shortly after transplantation. Therefore, excluding these two patients, the restoration rate of menses was 94% (47/50). Median FSH levels before AT were 63.8 IU/mL (IQR 38.5-90.3), and after AT, 19 IU/mL (IQR 10.7-36.5).

After transplantation, the patients were followed for a median duration of 64 months (IQR 36-82). During this time period, 33 of them underwent ovarian stimulation and IVF, 14 (42.4%) conceived at least once. A total of 117 cycles were completed, with a median three cycles per patient (IQR 2-5). In 25 of these cycles (21.36%), attempts to retrieve oocytes revealed empty follicles. One hundred and sixty-nine oocytes were collected and 88 were fertilized, yielding a fertilization rate of 52.07%. Altogether, 71 embryos were transferred. Mean oocyte number per cycle was 1.44, with clinical pregnancy and live birth rates per cycle of 14.5% and 12.8% respectively. Ultimately. A total of 17 pregnancies and 15 live births were obtained following IVF.

A total of 50 pregnancies were achieved (33 spontaneous, 17 IVF), 44 of which resulted in live births. Three of the pregnancies are still ongoing. Six patients conceived and delivered more than once; Four patients gave birth twice and 2 patients had 3 deliveries (one of them conceived 4 times and had spontaneous miscarriage). The overall miscarriage rate was 6%: 11.76% for IVF pregnancies and 3.03% for spontaneous pregnancies (p=0.29). Thirty patients conceived at least once (50%) and 25 gave birth at least once (41.6%). Two additional pregnancies were obtained in 2 out of 4 patients transplanted with vitrified tissue at the Infertility Center of St louis (12) but, as previously stated, these women were excluded from the analysis. Patients who conceived were compared to those who did not. Mean age on OTC was 25±5.5 vs. 27.6±6.8, respectively, but this difference did not reach statistical significance (p=0.1). However, there was a trend towards a higher proportion of women aged ≥35 years on OTC among those who did not conceive (53.3% vs 26.6%, p=0.06). In addition, a significantly younger age on AT was observed among those who became pregnant (31.6±5.2 vs 34.8±6.2, p=0.03). Hematologic malignancy was the most common diagnosis in both groups (70% vs 60%).

Among patients grafted with small tissue fragments, 58% conceived at least once as opposed to 46% of those transplanted with medium/large tissue fragments, but the difference was not statistically significant (p=0.41). The surgical approach did not impact outcomes; of those transplanted using a laparoscopic approach, 44.73% conceived at least once, compared to 55.26% of those undergoing AT by minilaparotomy (p=0.7). Of those whose tissue had been transplanted into ovarian site 51.5% conceived at least once, compared to 50% of those whose tissue had been transplanted into peritoneal pockets (=0.71).

Twenty-four women had their tissue stored after chemotherapy. Patients affected by hematologic malignancies were most likely to be exposed to chemotherapy before OTC (Figure 1). Table 2 shows transplantation outcomes according to chemotherapy treatment prior to OTC. Those who received and did not receive chemotherapy were of similar age. There was no significant difference between the two groups in terms of post-transplantation FSH values, restoration of menses, pregnancy, live birth, or miscarriage rates. Ten patients were treated with alkylating agents before OTC and exhibited no ovarian activity before AT. After AT, seven had their menses restored and four conceived at least once. The four patients who conceived were aged 23-32 years at OTC. One non-Hodgkin's lymphoma patient had been treated with 6 cycles of VACOP-B (etoposide, doxorubicin, cyclophosphamide, vincristine, prednisolone, bleomycin) before OTC and conceived via IVF following transplantation. The second, a Hodgkin's lymphoma patient, had been given 5 cycles of ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) and one cycle of MOPP (mechlorethamine, vincristine, procarbazine, prednisone) and conceived spontaneously after AT. The third, another Hodgkin's lymphoma patient, had undergone 6 cycles of BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone), conceived after two IVF attempts, and is currently 8 weeks pregnant. Finally, a breast cancer patient who received 4 cycles of AC (adriamycin and cyclophosphamide) and a further two cycles of cyclophosphamide, conceived after transplantation following just one IVF attempt.

Seven patients had their pelvis exposed to radiation prior to AT. All four leukemia/lymphoma survivors who underwent total body or lumbo-aortic radiation, and received pelvic radiation of up to 20Gy, regained

their menses following transplantation. Three of them conceived and delivered at term, with no obstetric complications, demonstrating preserved uterine function despite exposure to radiation. One colorectal cancer patient who was given pelvic radiation at a total dose of 45Gy also conceived after AT and replacement of a previously transposed ovary back to the pelvis. She delivered prematurely at 32 weeks of gestational age. By contrast, the two cervical cancer patients did not recover endocrine function and did not achieve pregnancy.

Twelve patients underwent more than one transplantation (Table 3); four of them underwent 3 ATs, and the remaining 8, 2 ATs. Mean age at OTC was 24.17 ± 4.62, while at first and last AT, it was 29.92 ± 3.84 and 32.83 ± 3.66 respectively. The median interval between transplantations was two years (range 1-5). All those who had their menses restored after the first transplantation also recovered menses following subsequent transplantations. In three patients, repeated ATs resulted in a total of 5 pregnancies and live births. Two of these women had also conceived following their first transplantation.

Discussion

The years 2004-2005 saw some groundbreaking advances in the world of reproduction. In 2004, the first live birth to be achieved spontaneously following OTC-AT was reported in Belgium (14). In 2005, the first live birth resulting from ovarian stimulation and IVF following OTC-AT in a woman with documented long-standing ovarian failure was announced in Israel (16). During that same year, a live birth obtained after transplantation of fresh ovarian cortex from a monozygotic twin was also recorded for the first time in the USA (19). In the 15 years that have passed since those transformative times, OTC-AT has gained ground as an accepted means of fertility preservation, practiced in multiple centers around the world on both an experimental and non-experimental basis. As of June 2017, it was estimated that OTC-AT had yielded more than 130 live births worldwide (20). This figure is expected to swell over the coming years, as the appeal of OTC-AT grows by virtue of its low surgical complication rates (21) and favorable outcomes. In a meta-analysis, OTC-AT was found to restore endocrine function in 64% of cases, with an ongoing pregnancy/live birth rate of 28.4% (10). Similar live birth rates are often observed when

scrutinizing data from other teams or networks, for example 32.6% in the Spanish group in 2018 (22), 31% in the Danish group in 2015 (9) and 25% in the German FertiPROTEKT network in 2016 (11).

Our first three reports of successful ovarian tissue transplantation from 2004-2005 triggered a learning curve that continues to this day, and the current paper presents the actual clinical findings of this ongoing process. Restoration of ovarian activity was observed in 94% of patients who had shown no ovarian activity prior to AT. At least one pregnancy was obtained in 50% of patients, and 41.6% of subjects delivered at least once. Such encouraging figures support use of OTC when fertility preservation is desired, emphasize its role in restoration of endocrine function and, importantly, advocate implementation of OTC as a non-experimental procedure. The positive results obtained in the current multicenter study may be attributed to extensive long-term experience in OTC-AT, as well as complete centralization of the entire process by each of the centers involved. When compared to other series in literature, a younger study population may account for the higher pregnancy rate observed in our study; the current study included a large number of hematologic patients who were at their 20's on presentation. In contrast, other series on literature included a higher proportion of breast cancer patients, who underwent OTC at an older age (9, 11, 22). Substantiates this explanation is a trend towards a higher proportion of patients aged ≥35 on OTC among those who did not conceive in our cohort. Age differences could also explain the low miscarriage rate observed in this study (6%), as opposed to the one described by other groups (~33%) (9, 22)..

It is still unclear whether OTC is preferable to oocyte cryopreservation in postpubertal patients who have sufficient time for ovarian stimulation before chemotherapy. A study comparing the two techniques reported a trend towards higher pregnancy (40.8% vs 32.6%) and live birth (27.3% vs 18.2%) rates in patients undergoing oocyte vitrification (22). However, it is noteworthy that the favorable outcomes of oocyte vitrification presented in the study above are from fertility centers with vast experience in oocyte freezing. On the other hand, as previously stated, a number of groups around the world have achieved live birth rates of more than 18% following OTC-AT, and the results of the current analysis considerably outperform this figure. Moreover, OTC has several advantages over oocyte vitrification. It restores ovarian

function and enables natural conception, as reflected by the 33 spontaneous pregnancies achieved in this series (leading to a spontaneous clinical pregnancy rate of 55%). Another advantage demonstrated by the current cohort is that OCT-AT may result in several pregnancies. These are less likely to occur following a single cycle of IVF for oocyte cryopreservation. When possible, a combination of OTC and oocyte cryopreservation can be offered to further increase the chances of future pregnancy (23).

Importantly, ovarian stimulation and oocyte retrieval become irrelevant immediately after chemotherapy, and for at least another 6 months (8), resulting in a diminished response to ovarian stimulation (24, 25) and genetically abnormal oocytes, as demonstrated in animal models (26-28). Hence, OTC is the only fertility preservation option available to patients who cannot postpone chemotherapy upon primary diagnosis or disease relapse. There is still ongoing debate around the efficacy and feasibility of OTC in the context of previous chemotherapy, with some centers restricting the procedure to chemotherapy-naive patients only (29). However, in the present cohort, both chemotherapy-naive and chemotherapy-exposed patients showed similar ovarian activity restoration rates of about 94%. The proportion of women who conceived and delivered at least once was actually slightly higher among chemotherapy-exposed patients (58% vs 42% and 46% vs 39% respectively) despite being of similar age, but this difference was not statistically significant. These results corroborate those observed in a smaller recent study reporting outcomes of 31 autotransplanted patients (30). By one year after AT, ovarian function was restored in 93% of chemotherapy-exposed subjects as opposed to 67% of non-exposed subjects ($p=0.14$). None of the chemotherapy-naive patients conceived, while 32% of the chemotherapy-exposed group delivered at least once, although lower pregnancy rates were encountered among patients treated with alkylating agents. In our study, however, 7 out of 10 subjects previously exposed to alkylating agents regained ovarian function following AT. Four of them conceived and gave birth, despite exposure to cyclophosphamide. Due to a lack of statistical power, the findings of the present study cannot refute a possible decline in AT outcomes in case of preharvesting chemotherapy exposure, especially when alkylating agents are involved.

Nevertheless, chemotherapy-exposed patients did not show inferior results to chemotherapy-naive patients . Withholding OTC-AT from chemotherapy-exposed patients therefore seems unjustified.

The value of AT in patients with previous exposure to pelvic radiation is unclear. Assuming orthotopic transplantation is performed, poor pelvic vascularization may compromise graft function, making the procedure somewhat ineffective. Importantly, even if ovarian function is preserved or restored, radiation-induced uterine injury may impede uterine capability to carry a pregnancy, so surrogacy is often required. Our data suggest that AT may be appropriate in patients whose pelvis is exposed to radiotherapy, as four out of 5 patients receiving pelvic radiation at doses ≤ 45 Gy conceived and delivered. While patients who received radiation at doses ≤ 20 Gy delivered on term without any complications, the patient who was treated with a higher dose of 45 Gy delivered prematurely at 32 weeks. A higher dose of pelvic radiation (external and brachytherapy) is given in cases of cervical cancer (31), and none of the cervical patients in our cohort managed to conceive. to the best of our knowledge, there have been no reports of successful transplantation in such cases. When contemplating fertility preservation in a patient destined to undergo pelvic radiotherapy, informed discussion is essential. Limited experience with the procedure in such circumstances should be communicated to the patient, as should the possibility of alternative options and potential need for surrogacy in the future. A higher risk for numerous obstetric complications should be conveyed to the patient as well, including miscarriage, preterm labor and intra-uterine growth restriction (32). If AT is pursued, tissue should be grafted to a vascularized site, such as an ovary replaced back inside the pelvis after transposition.

Graft dimensions have been shown to play a significant role in determining the extent of follicle loss during cryopreservation, thawing and transplantation. Upon freezing, thinner cortical strips allow good penetration of cryoprotective agents and decrease the likelihood of ice crystal formation and injury (33). Upon transplantation, smaller dimensions reduce penetration depth for new blood vessels, potentially shortening the ischemic period (34, 35). On the other hand, grafting smaller fragments may boost follicle activation, resulting in increased loss of dormant follicles (36). The present study has enabled us to describe AT clinical

results according to fragment dimensions for the first time. Patients transplanted with small tissue strips ($\leq 9mm^2$) achieved similar pregnancy and live birth rates to those transplanted with larger pieces. Tissue dimensions may determine graft's life span, but such hypothesis could not be evaluated in the current study. In order to determine optimal tissue strip dimensions, if they exist at all, larger multicenter studies, also evaluating grafts' life span, should be conducted. As experience with OTC and transplantation continues to grow globally, such studies will undoubtedly be available in the near future.

In the event of graft malfunction, repeat AT can be considered if a sufficient amount of ovarian tissue was stored. To prevent complications of repeated surgical procedures, it is important to identify patients most likely to benefit from additional transplantation. In the present study, 12 patients underwent more than one AT. All those whose menses resumed following their first transplantation also exhibited ovarian activity after their subsequent transplantation. By contrast, the only woman not to recover her menses following her first transplantation did not show any ovarian activity after her second transplantation either. Repeat ATs yielded a total of 5 live births in 3 patients, two of whom had been transplanted for the second or third time after already giving birth following their first AT. These patterns may suggest that first and repeat transplantations share a similar course and outcomes. Hence, patients who manage to initially conceive following AT, but later experience diminished/absent graft activity, may be encouraged to pursue a second transplantation if an additional pregnancy is desired.

Several strength and limitations of the study should be acknowledged. Among the strengths of the current study is the centralization practiced by the three participating centers, exclusively managing every case through all aspects of patient care, from OTC to AT and subsequent follow up. Additionally, to the best of our knowledge, our study presents the largest series to evaluate the effect of chemotherapy exposure on AT outcomes, and is the first to describe pregnancy rates according to different surgical strategies. Yet, due to the limited years of OTC-AT practice combined with a low return rate of 5.3%, and despite the integration of data coming from three centers, the modest number of patients who underwent AT could limit the sensitivity of statistical tests. The effect size observed when comparing technical strategies was small,

limiting the statistical power to detect differences in pregnancy rates. The return rate in the current cohort is rather low, but nevertheless comparable to utilization rates observed by other groups, in oocyte vitrification(22), OTC-AT(11, 22) and male fertility preservation programs (37, 38). Improved identification of those likely to utilize their tissue is of great importance and should be addressed in future studies.

Conclusion

This multicenter study reinforces the role of OTC as an effective tool for fertility preservation, resulting in conception and live birth in up to 50% and 41.6% of patients respectively. Our study reveals that once a previously functioning graft is depleted, repeat AT is likely to trigger endocrine activity and conception once again. OTC is highly appropriate in case of recent chemotherapy and should not be excluded in such scenarios. AT following pelvic exposure to radiation can yield satisfactory results in selected cases, not always requiring surrogacy. However, the efficacy of AT remains questionable in case of cervical cancer. In terms of techniques used when handling harvested tissue, neither tissue dimensions nor the surgical approach to transplantation appear to affect reproductive outcomes, but these should be further evaluated in larger studies in the future. It is expected that in the coming years, implementation of OTC-AT will continue to expand globally, with increasing numbers of procedures performed for non-oncologic indications too. We can therefore expect a growing body of data to accumulate in the near future, allowing for large-scale studies to better understand and address the impact of technical aspects and selection criteria on outcomes, further improving efficiency and performance. In the meantime, it is fair to say that in the hands of experienced practitioners, OTC-AT is a highly effective approach to fertility preservation.

Table 1- Surgical methods practiced by each participating center

	Sheba Medical center	Cliniques Universitaires Saint-Luc	Infertility Center of St. Louis
Number of transplanted patients (number of ATs)	34 (39)	25 (34)	11(14)
Number of eligible patients[1] (number of ATs)	32(37)	23(32)	5(7)
Tissue Fragments dimensions (median)	$50mm^2$ (IQR 50-50, range 2-60)	19.5 mm^2 (IQR 7-45, range 4-64)	$50mm^2$ (IQR 50-50, range 36-50)
Number of fragments transplanted (median)	4 (IQR 3.8-6, range 2-20)	13 (IQR 5.1-22, range 3-70)	5 (IQR 2.8-7.5, range 2-9)
AT surgical approach	Minilaparotomy - 32/37 Laparoscopy - 5/37	Minilaparotomy - 3/32 Laparoscopy - 29/32	Minilaparotomy -7/7
Graft site	Ovary-subcortical pockets- 22/37 Peritoneal window- 1/37 Both -14/37	Ovary-denuded medulla- 18/32 Peritoneal window- 5/32 Both- 9/32	Ovary-denuded medulla -7/7

AT: autotransplantation
IQR: interquartile range
1- Patients whose tissue was vitrified and those who were harvested due to premature ovarian insufficiency were not included. See additional exclusion criteria on text.

Table 2 – Ovarian transplantation outcomes according to pre-harvesting chemotherapy exposure

	Total	Chemotherapy	No chemotherapy	p-value
Patients	60	24	36	
Age at OTC (years)	26.3 ± 6.26	25.83 ± 5.65	26.55 ± 6.71	0.66
Age at AT (years)	32.7 ± 5.6	32.96 ± 6.1	33.36 ± 5.64	0.80
Tissue transplanted (area, mm^2)	250 (150-300)	200 (150-214)	200 (165-310)	0.11
FSH after AT (IU/mL)	19 (10.7-36.5)	14 (8.8-42.5)	23.65 (12-34.2)	0.26
Menses recurrence[1]	(47/50) 94%	(19/20) 95%	(28/30) 93.3%	0.81
At least one pregnancy	(30/60) 50%	(14/24) 58.33%	(16/36) 44.4%	0.32
At least one live birth	(25/60) 41.66%	(11/24) 45.83%	(14/36) 38.9%	0.79
Number of pregnancies (IVF)	50 (17)	25 (8)	25 (9)	
Number of live births (IVF)	45 (15)	21 (5)	24 (10)	
Number of ongoing pregnancies (IVF)	3 (1)	1 (1)	2 (0)	
Miscarriages	(3/50) 60%	(3/25) 12%	(0/25) 0%	0.24

1- Ten patients were excluded from the analysis: eight patients who had residual ovarian activity prior to transplantation (N=8), one patient who underwent hysterectomy, and an additional patient who was treated with chemotherapy after being diagnosed with secondary malignancy shortly after transplantation

OTC: ovarian tissue cryopreservation
AT: autotransplantation
FSH: follicle-stimulating hormone
IVF: in vitro fertilization

1

2

Table 3- Repeat ovarian tissue transplantations- patients characteristics and results

			OTC			Before AT			After AT			
Patient #	AT#	Diagnosis	Age	Chemotherapy regimen	Radiation	Age	Menses	FSH (IU/mL)	Menses	Time to menses	FSH (IU/mL)	Pregnancies/ live births
1	1	CML	21	/	TBI	27	no	116	yes	3M	7.2	
	2					28	no	44	yes	3M	100	
	3					33	no	50	yes	3M	14	
2	1	Ewing sarcoma	24	/	/	28	no	107	yes	3M	24.3	1/1 (IVF)
	2					31	no	80	yes	2M	9	1/1 (IVF)
3	1	Hodgkin's lymphoma	21	ABVD	/	28	no	71	yes	8M	21.5	
	2					29	no	35	yes	0.5M	31	
4	1	Non-Hodgkin's lymphoma	36	RCHOP	/	40	no	87	no	/	90	
	2					41	no	90	no	/	78	
5	1	Hodgkin's lymphoma	25	/	/	30	no	91	yes	4M	34	
	2					31	yes	34	yes	4M	NA	1/1 (spontaneous)
6	1	Sickle cell disease	20	/	/	26	no	44	yes	5M	22	
	2					27	yes	25	yes	4M	NA	
	3					31	yes	NA	yes	4M	25	
7	1	Non-Hodgkin's lymphoma	28	ABVD	/	34	no	65	yes	6M	80	
	2					36	yes	80	yes	2M	78	
8	1	Wegener's granulomatosis	22	/	/	27	no	114	yes	5M	60	
	2					28	yes	60	yes	4M	IVF	
9	1	Hodgkin's lymphoma	23	ABVD	/	30	no	72	yes	6M	103	
	2					33	yes	103	yes	5M	64	
	3					35	yes	64	yes	2M	NA	
10	1	Hodgkin's lymphoma	22	MOPP/ ABVD	/	28	yes	62	yes	6M	118	
	2					30	yes	41	yes	NA	NA	
11	1	Hodgkin's lymphoma	28	/	/	30	no	50	yes	9M	48	
	2					33	no	48	yes	6M	35	
12	1	Hodgkin lymphoma	20	ABVD	TBI	31	no	64.2	yes	4M	8.2	1/1 (Spontaneous)
	2					33	no	62.4	yes	4M	3.9	
	3					36	no	61.2	yes	4M	5	3/3 (Spontaneous)

OTC: ovarian tissue cryopreservation
AT: autotransplantation
FSH: follicle-stimulating hormone
CML: chronic myeloid leukemia
ABVD: doxorubicin, bleomycin, vinblastine, dacarbazine
MOPP: mechlorethamine, vincristine, procarbazine, prednisone)
RCHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone
TBI: Total body irradiation

Figure legends

Figure 1. Chemotherapy exposure according to diagnosis. Hematologic malignancies were more commonly associated with chemotherapy before ovarian tissue cryopreservation.

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Revision notes

We would like to thank the Editorial Manager of *Fertility & Sterility* and the Reviewers for the time, efforts and serious dedication to review our study, as well as for the constructive comments and suggestions. The peer review process has helped to improve the quality of our manuscript.

We have addressed all the comments of the Reviewers and revised the manuscript accordingly. We hope that the revised version of the manuscript is considered suitable for publication in *Fertility & Sterility.* An itemized reply to the comments of the Reviewers is provided below.

Reviewer #2:

The manuscript has improved considerably after revision. I have just one additional comment: In the text you write that all patients were offered OTC prior to a potentially sterilizing treatment. And yet in Table 2 you state that the majority of the patients (N=36) had NOT received any chemotherapy before OTC and you also state in the text that 7 had received radiation therapy. I suppose all of the 7 patients also received chemotherapy, so the question is why these 36 patients received OTC??

Response: We are sorry if we weren't clear on this issue. All of the patients included underwent OTC as a fertility preservation measure, before being treated with potentially sterilizing chemotherapy (=**post** harvesting chemotherapy). These patients can be further divided into two groups: those treated with chemo before OTC (=**pre** harvesting chemotherapy, N=) and those who were not. In other words, all of the patients were treated with post harvesting chemotherapy, but only some of them were treated with preharvesting chemotherapy. We changed the title of table 2 accordingly, we hope that it is clearer now.

Editorial comments:

1. Include mailing address for corresponding author.

Response: included in the first page

2. Revise capsule to be a complete sentence(s) of 30 words or less.

Response: revised as requested

3. In your abstract, please rephrase the Objective to begin, "To

Response: rephrased as requested

4. Include 3-5 keywords after the abstract.

Response: added as requested

Dear editor,

We are pleased to submit our manuscript entitled: **"Critical evaluation of ovarian tissue transplantation results from three centers having more than 15 years' experience"** for consideration as an original article.

In recent years, ovarian tissue cryopreservation has gained grounds as an established technique for fertility preservation which is increasingly applied worldwide. Yet, there is inconsistency regarding selection criteria for the procedure, laboratory handling of harvested tissue, and the transplantation technique itself. Thus, there is a growing need to better define selection criteria and determine the optimal technical approach.

This manuscript presents the combined results of 70 ovarian auto-transplantations performed on three different centers, all with more than 15 years of experience in tissue transplantation. The current study takes into account the different strategies of each of the centers in order to provide generalizable success rates and critically investigate the relationship between key technical/clinical factors and procedure outcomes.

This manuscript has not been previously published and is not under consideration in the same or similar form in any other peer-reviewed media. All authors listed have contributed sufficiently to the project to be included as authors. To the best of our knowledge, no conflict of interest, financial or other, exists.

Sincerely,

Moran Shapira MD

Dror Meirow MD

Figure(s)

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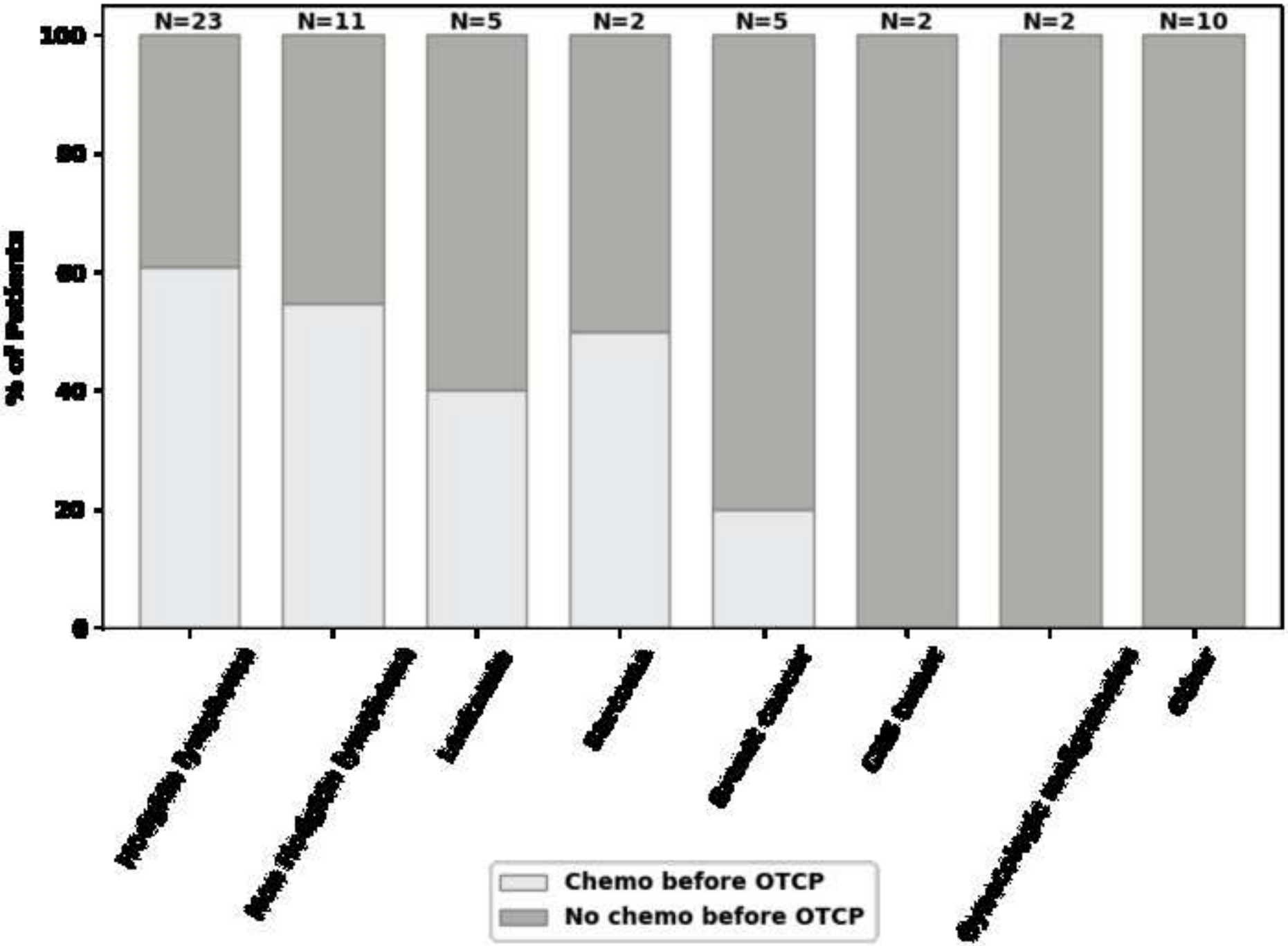

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Date: 09/10/2019 **Manuscript # (if available):** _____

Manuscript title: **Critical evaluation of ovarian tissue transplantation results from three centers having more than 15 years' experience**

Corresponding author: **Dror Meirow**

Authors may either sign the same form or submit individually

I am an author on this submission, have adhered to all editorial policies for submission as described in the Information for Authors, attest to having met all authorship criteria, and disclosed all potential conflicts of interest for inclusion on the title page of the submission.

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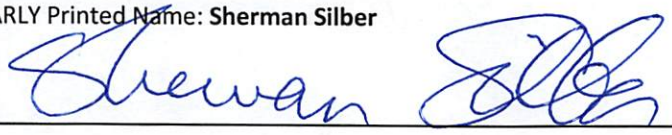
Signature:

Typed or CLEARLY Printed Name: **Sherman Silber**

Signature: 

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Signature:

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Each author is required to submit a signed Statement of Authorship upon submission. This applies to all submission types including Editorials, Letters to the Editor, etc.

Date: 09/10/2019 **Manuscript # (if available):** _____

Manuscript title: Critical evaluation of ovarian tissue transplantation results from three centers having more than 15 years' experience

Corresponding author: Dror Meirow

Authors may either sign the same form or submit individually

I am an author on this submission, have adhered to all editorial policies for submission as described in the Information for Authors, attest to having met all authorship criteria, and disclosed all potential conflicts of interest for inclusion on the title page of the submission.

Signatures are required - typed signatures are unacceptable.

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Signature:

Typed or CLEARLY Printed Name: **Marie Madeleine Dolmans**

Signature:

Typed or CLEARLY Printed Name: **Sherman Silber**

Signature:

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Signature:

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*Completed STROBE checklist

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract [p. 3]
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found [p.3]
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported [p.4]
Objectives	3	State specific objectives, including any prespecified hypotheses [p.4]
Methods		
Study design	4	Present key elements of study design early in the paper [p. 5]
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection [p. 5-6]
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up [p.5-7]
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable [p. 5-6]
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group [p.5]
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at [p. 5]
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why [p.8]
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding [p.8]
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how loss to follow-up was addressed
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed [p.8]
		(b) Give reasons for non-participation at each stage [p.8]
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders [p.8-9]
		(b) Indicate number of participants with missing data for each variable of interest [
		(c) Summarise follow-up time (eg, average and total amount) [p.8]
Outcome data	15*	Report numbers of outcome events or summary measures over time [p 8-10, table 2]
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included

		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses [table 2]
Discussion		
Key results	18	Summarise key results with reference to study objectives [p 10-11]
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias [p. 12]
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence [p. 12-14]
Generalisability	21	Discuss the generalisability (external validity) of the study results [p.11]
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based [p.1-2]

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.