In vitro study on the effect of double freezing on ovarian tissue preservation in cancer women

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Ovarian tissue cryopreservation (OTC) is very debated in women with breast cancer, chemotherapy and mutations in breast cancer 1 and breast cancer 2 (BRCA) genes, due to the high risk of developing ovarian cancer after ovarian tissue reimplantation. Objective: The present study aims to evaluate the effects of a double slow freezing/rapid thawing procedure on the quality of human ovarian tissue, in terms of preservation of tissue components and follicle viability by light microscopy. Methods: To this end the ovarian tissue of three women, donated for research purposes, was subjected to a single (T1) and double (T2) OTC procedure. For each woman fresh (CTRL-T0), T1 and T2 samples were fixed in formaldehyde for light microscopy analysis. In addition, for T1 and T2 samples of each woman an additional sample was thawed, in vitro cultured for 4 hours (h) and subsequently subjected to neutral red staining combined with histological evaluation.

Results: After re-freezing, a good preservation of follicles and stroma was observed by histological and immunohistochemical (Ki67 and Bcl2) analysis. Moreover, a high follicle survival rate resulted after in vitro culture and Neutral Red staining followed by histological evaluation.

Discussion: Although the analyzed tissue was not reimplanted in human or/and animal models, these preliminary data, suggest the possibility of twice freezing ovarian tissue as a potential new way to treat women with breast cancer at risk of recurrence or ovarian cancer. In these women to safeguard the women’s health, the transferred ovarian tissue could be removed after childbearing and re-frozen for later motherhood request.

Keywords
Ovarian tissue cryopreservation, Breast cancer, BRCA-mutated breast cancer women, Light microscopy

1. Introduction
Breast cancer (BC) is the tumor most commonly found in premenopausal women with a prevalence of 10.5% [1]. Most BCs are sporadic, while 5–10% are due to a hereditary predisposition [2]. Autosomal dominant mutations in breast cancer 1 and breast cancer 2 (BRCA) genes, probably cause 3–4% of all BCs and most hereditary cases of early-onset BC [3]. Recent advances in cancer diagnosis and the use of new chemotherapy protocols have significantly increased survival rates for women with BC [1]. However, these treatments are gonadotoxic and can severely reduce, if not completely destroy, the reproductive potential of these premenopausal women by inducing premature ovarian failure [4]. A recent meta-analysis reported that 40% to 50% of women want to have a family after their BC treatment [5]. Dealing with cancer is inherently strenuous and is particularly difficult if the treatments needed for healing may impair the reproductive sphere and the ability of a normal and complete life. Therefore, women with BC and BC BRCA mutation carriers of reproductive age should be informed at the time of diagnosis about the potential negative effects of cancer therapy and the chances of fertility preservation. Embryo or oocyte cryopreservation is usually the first option for these women [6, 7]. Both procedures require ovarian stimulation, which may take two weeks to complete, leading to a delay in the start of therapies and significantly increases estrogen levels, which is undesirable in most women with hormone-sensitive BC. Egg retrieval is also possible in a natural cycle, but this procedure has a very low yield of oocytes and embryos [8]. Therefore, in these cases the ovarian tissue cryopreservation (OTC) may represent a valid option to preserve the ovarian function, since it can be performed on any day of the menstrual cycle, avoiding delays in cancer treatment and not requiring ovarian stimulation. After remission of the disease in presence of POF or regular menstrual cycles but limited ovarian reserve [9], the cryopreserved ovarian tissue can be reimplanted to restore endocrine (90–100% of cases) and reproductive activity (over 130 healthy children worldwide) [4, 10–14]. Despite the positive outcomes, the use of OTC is much debated in women with BRCA-mutated BC, due to the high risk of developing ovarian cancer after ovarian tissue reimplantation. To protect women from the risks of re-developing cancer,
the reimplanted ovarian tissue could be removed after the baby is born, frozen for the second time and potentially transplanted for a future desire for motherhood.

The present study aims to evaluate the effects of a double slow freezing/rapid thawing procedure on the quality of human ovarian tissue, in terms of preservation of tissue components and follicle viability by light microscopy.

2. Materials and methods

2.1 Subjects

The study was performed on the ovarian tissue of three women with breast cancer aged 25, 27 and 28 years (26.7 ± 1.5 years, mean ± standard deviation [SD]) who cryopreserved ovarian tissue before chemotherapy at our Center. Before OTC all women were subjected to the assessment of endocrine parameters and biometric measurements, showing normal ovarian aging matched by age and any endocrine signs of premature menopause (FSH 5.4 ± 0.8 mIU/mL; LH 5.9 ± 0.3 mIU/mL; E2 42.3 ± 5.4 pg/mL; P 0.48 ± 0.2 ng/mL; AMH 1.8 ± 1.1 ng/mL).

2.2 Cryopreservation and study design

The cryopreservation procedure was approved by our local Ethics Committee (S. Orsola-Malpighi Hospital, No. 74/2001/O). For each woman, an ovarian biopsy was performed by laparoscopy. After medulla cleaning, the ovarian cortex was cut into slices 1.5 × 0.5 × 0.2 cm and cryopreserved using the slow freezing/rapid thawing protocol described by Fabbri et al. [15]. A fresh sample (0.5 × 0.2 cm) (CTRL-T0) was fixed in formaldehyde for light microscopy analysis. Ten years after OTC, women donated ovarian tissue for research. An ovarian slice of each woman was thawed and divided into two parts: a sample (0.5 × 0.2 cm) (T1) was subjected to the same analysis of T0, while the remaining part of the slice (1 × 0.2 cm) was slowly frozen for a second time. After a week of storage, the sample (T2) was thawed and processed for light microscopy. The study design is reported in Fig. 1. For T1 and T2 samples of each woman an additional sample was thawed, in vitro cultured for 4 hours (h) and subsequently subjected to neutral red staining combined with histological evaluation.

2.3 Histological and immunohistochemical analysis

T0, T1 and T2 samples were embedded in paraffin blocks and cut in 4 μm thick sections. Every 50 μm, a section was collected: the first, third and fifth were stained with hematoxylin and eosin (Merck, Darmstadt, Germany) to assess the follicle number and the morphological features of follicles and stromal cells. The second, fourth and sixth sections were used for immunohistochemistry as described in Fabbri et al. [16] and incubated overnight at 4 °C with the following primary antibodies: anti-Ki67 (1 : 80, BioGenex, San Ramon, CA, USA), anti-Bcl2 (1 : 80, Dako, Carpinteria, CA, USA). An En Vision monoclonal immunoenzymatic system was used for immunohistochemical detection (Dako). The reaction was developed in 3,3-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA). Finally, the sections were counterstained with hematoxylin for 10 seconds, dehydrated and mounted in Eu-kitt. The control procedures were undertaken simultaneously to ensure the specificity of immunostaining. Sections without primary antibodies were used as negative control and sections of human BC were used as positive control.

2.3.1 Histological and immunohistochemical evaluation

The samples were examined by a pathologist using the Leitz optical microscope equipped with a camera, in order to assess the degree of preservation of the frozen-thawed ovarian tissue. The sections were observed with a 100× magnification, to identify any artifacts and the presence of follicles, then observed with a 250× magnification to establish the development stage of follicle, according to Gougeon (1996) [17] classification, and the preservation of follicles and stroma. For each woman, the number of follicles was counted in all the analyzed sections and then mediated.

The degree of follicular and stromal preservation was determined using the method previously described by Fabbri et al. [18]. Follicle preservation was expressed as number of preserved follicles/total number of counted follicles × 100.

The immunopositivity of the follicles and stroma was assessed with a 250× magnification. Follicles with stained oocyte nucleus or and a granulosa cell were considered pos-
Fig. 2. Histological and immunohistochemical analyses. (A, B, C) control samples of ovarian tissue (T0); (D, E, F) samples of ovarian tissue after first freezing/thawing (T1); (G, H, I) samples of ovarian tissue after second freezing/thawing. Hematoxylin/eosin staining (A, D, G). Immunohistochemical staining for Ki67 (B, E, H) and for Bcl2 (C, F, I).

itive. The immunopositive stromal cells were counted three times in an area of 100 mm$^2$ (randomly selected in the section). The images were analyzed with the Image ProPlus software.

2.4 Neutral red staining

Follicle viability was assessed by Neutral Red (NR), a dye that in human ovarian cortex stain only viable follicles, whereas dead follicles remain un-stained [19].

The thawed cortical tissue pieces were treated as reported by Kristensen et al. [20]. Briefly, after single and double OTC procedure, tissues were transferred into a 60 mm tissue culture dish and incubated for 4 h at 37 °C and 5% CO$_2$ in 8 mL of αMEM (Biowest) supplemented with 20% human serum albumin (HSA, FujiFilm, Irvine Scientific), 2 mM L-glutamine, 100 UI/mL penicillin-G, 0.1 mg/mL streptomycin, ITS liquid media supplement and 50 µg/mL NR solution (N2889) (all from Sigma-Aldrich). Tissue pieces were incubated with NR in situ for 4 h at 37 °C and 5% CO$_2$. Tissue samples containing NR-stained follicles were identified under a stereomicroscope (Leica) equipped with a DS-Fi1 camera (Nikon). A NR-stained follicle count was recorded for each tissue sample. Each sample was subsequently histologically processed in order to provide the total number of follicles, which allowed the calculation of the follicle survival rate for cryopreserved tissue.

2.5 Statistical analysis

The data were analysed with GraphPad Prism (software version 7.0, San Diego, CA, USA). The results were represented as mean ± SD and P value was determined by one-way analysis of variance and Bonferroni post-hoc test. Differences with P < 0.05 were considered statistically significant.

3. Results

The ovarian samples (T0-CTRL) of all cases were histologically well preserved. In particular, 80, 97, 85 follicles were counted and analyzed for cases 1, 2 and 3, respectively. In all samples, most of follicles were primordial (97 ± 4.5%) and the remaining (3 ± 1.2%) were represented by follicles at an advanced stage of development (primary, secondary,
pre-antral). The follicles showed a regular shape and a good adherence between the granulosa cells and oocyte; the latter was characterized by a roundish nucleus with dispersed chromatin and granular cytoplasm (Fig. 2A). The percentage of preserved follicles resulted 67.5% for the case 1, 63.2% for the case 2 and 70.1% for case 3. The stroma of the samples in all cases had collagen fibers organized in vortices, cells with dispersed chromatin. In the cytoplasmic stromal compartment, vacuoles and interstitial edema were not appreciable (Fig. 2A).

Immunohistochemical analysis showed a similar staining pattern in all cases. In particular, a nuclear positivity for the proliferative antigen Ki67 was observed in the oocytes of the primordial follicles and in the granulosa cells of the growing follicles (Fig. 2B). Regarding the anti-apoptotic protein Bcl2, positive staining was found in the cytoplasm of the granulosa and stromal cells (Fig. 2C).

After the first thawing (T1), 52, 48 and 57 follicles were analyzed for cases 1, 2, and 3 respectively. A significant reduction ($P < 0.05$) in the number of follicles was found between T0 and T1 for all cases. In all samples, 98 ± 2.7% of follicles were primordial, while the remaining 2 ± 1.4% were primary and secondary follicles. The ovarian tissue in all cases showed oocytes with morphological characteristics (Fig. 2D) comparable to those observed at T0. The percentage of preserved follicles resulted 72% for the case 1, 68% for the case 2 and 69% for the case 3 (T1 vs T0, $P = NS$). The stroma also appeared to be well preserved (Fig. 2D). Similarly to T0, a positive nuclear staining for Ki67 was observed in oocytes and granulosa cells of growing follicles (Fig. 2E) and a diffuse positive staining for Bcl2 in the cytoplasm of granulosa cells and stroma (Fig. 2F).

After the second thawing (T2), 60, 46 and 52 follicles were analyzed for cases 1, 2, and 3 respectively. The number of follicles observed in T2 was significantly lower than T0 ($P < 0.05$), while no difference was found between T2 and T1 ($P = NS$). In all samples, 97 ± 3.8% of follicles were primordial, while the remaining 3 ± 3.2% were primary and secondary follicles. The ovarian tissue in all cases showed oocytes with morphology (Fig. 2G) similar to T0 and T1. The percentage of preserved follicles resulted 61.7% for the case 1, 62.5% for the case 2 and 62.7% for the case 3 (T2 vs T0 and T2 vs T1, $P = NS$). The stroma also appeared to be well preserved (Fig. 2G). The Ki67 (Fig. 2H) and Bcl2 (Fig. 2I) immunostaining was similar to that observed in T0 and T1.

For all cases the number of follicles and the percentage of preserved follicles in each experimental condition are reported in Table 1.

As regard follicle viability, NR-stained follicles were present either in T1 and T2 tissue samples of all women (Fig. 3). The average of NR-stained follicles counted per woman and the overall follicle survival rate in ovarian tissue from the three women after single and double OTC procedure are reported in Table 2. For all cases, no statistical differences were observed in the number of NR-stained follicles and in the survival rate at T1 and T2 ($P = NS$).

4. Discussion

Performing a double freezing of ovarian tissue could open new perspectives for the OTC application in women in whom ovarian tissue reimplantation is not recommended because of the risk of recurrence or ovarian cancer. In these women the ovarian tissue could be reimplanted to restore ovarian function and allow a pregnancy, after which it could be removed and re-frozen for a second desire for motherhood. In this context, it is very important to evaluate the effect of a double freezing/thawing procedure on the quality of preservation of ovarian tissue. Several studies reported a reduced "reproductive potential" after transplantation of frozen ovarian tissue, compared to that obtained using fresh tissue [21–24]. This could be related to the damage that the ovarian tissue can suffer during the cryopreservation phases [25–29]. In fact, OTC is a complex procedure that requires technical experience and protocol validation [18, 30, 31]. Over the past 16 years, our group [15, 16, 18, 32] optimized the cryopreservation protocol by studying: (a) the seeding temperature, which is a crucial step of the slow freezing protocol; (b) cryoprotectant concentrations; (c) protein support and (d) temperature of thawing procedure. In the present study, we evaluated the effect of the double optimized cryopreservation protocol on human ovarian tissue, using the morphological analyses, as already previously reported [15, 16]. Histological evaluation showed good preservation of all ovarian components after the first and second freezing/thawing, comparable to those observed in the control samples, confirming the effectiveness of the cryopreservation procedure. The proliferative state of the ovarian cell population was also assessed by the Ki67 immunostaining. The Ki67 antibody recognizes a nuclear antigen which is expressed throughout the stages of the cell cycle except G0, is therefore a good indicator of tissue cryopreserved ability to proliferate after thawing. A positive staining was found in most follicles, suggesting that they could resume the meiotic cycle and subsequently grow. The high percentage of positivity for Bcl2 in granulosa and stromal cells is a further confirmation of the good preservation of the ovarian tissue after standard cryopreservation procedure.

### Table 1. Number of follicles and percentage (%) of preserved follicles in human ovarian tissue subjected to a single and double OTC procedure. Data from 3 patients are reported.

| Patient | Age (yrs) | Follicles (N°) | Preserved follicles (%) |
|---------|-----------|----------------|------------------------|
|         |           | T0 | T1 | T2 | T0 | T1 | T2 |
| 1       | 25        | 80 | 52 | 60 | 67.5 | 72 | 61.7 |
| 2       | 27        | 97 | 48 | 46 | 63.2 | 68 | 62.5 |
| 3       | 28        | 85 | 57 | 52 | 70.1 | 69 | 62.7 |

$a P < 0.05.$

T0, control ovarian tissue samples; T1, ovarian tissue samples after the first freezing/thawing; T2, ovarian tissue samples after the second freezing/thawing.

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Fig. 3. Follicle viability by NR-staining. NR-stained follicles present within the thawed ovarian tissue after 3–4 h in situ incubation. (A) T1: ovarian tissue sample after the first freezing/thawing; (B) T2: ovarian tissue sample after the second freezing/thawing. NR, Neutral red.

Table 2. Number of Neutral red (NR) stained follicles, number of follicles identify by histology and percentage of follicles survival rate in human ovarian tissue subjected to a single and double OTC procedure. Data from 3 patients are reported.

| Patient | N° of NR-stained follicles | N° follicles (Histology) | Follicle survival rate (%) |
|---------|---------------------------|-------------------------|---------------------------|
|         | T1 | T2 | T1 | T2 | T1 | T2 |
| 1        | 47 | 46 | 69 | 77 | 68 | 60 |
| 2        | 36 | 27 | 53 | 52 | 67 | 52 |
| 3        | 51 | 49 | 82 | 87 | 62 | 56 |

T1, ovarian tissue samples after the first freezing/thawing; T2, ovarian tissue samples after the second freezing/thawing.

The number of follicles decreased after freezing/thawing compared to the control, while it remained constant between the first and second freezing/thawing. To assess the follicle survival rate after a double OTC, an in vitro culture followed by NR staining combined with histological evaluation was performed. The vital dye NR accumulates in viable follicles allowing to estimate the number of viable follicles within the tissue. NR is a commonly used assay to determine the viability of primordial follicles in vitro and it has been proposed as a validation tool to assess freezing protocols when clinics start a program for OTC and as a routine quality control for the overall freezing performance within the tissue banking facility, although it cannot replace the xenografting model [20]. In the present study a high follicle survival rate resulted after a double OTC, supporting the validity of the procedure.

An casual distribution of follicles emerges in the examined samples. In adult women follicles are reduced in number and organized in cluster. Therefore, a different follicle number can be found in adjacent samples of a slice. Also, the follicle reduction, from their recovery to thawing, could be attributed to mechanical injury during tissue preparation and/or to the cryopreservation procedure. However, it is worth mentioning that the most vulnerable follicles to freezing/thawing are the largest follicles than primordial ones [33–35]. Moreover, studies comparing fresh and frozen-thawed transplanted tissues show similar rates of follicle survival [36] and functioning [37] which reinforces the hypothesis that cryopreservation does not significantly interfere with the function of primordial follicles in tissue grafts [35], as confirmed by our outcomes. To date, only one study [38] reported a successful case of double OTC freezing. A 23-year-old woman with mucinous ovarian cystadenocarcinoma underwent OTC of the contralateral ovary, subsequent reimplantation and successful in vitro fertilization treatments, with the birth of two healthy babies. To safeguard the woman’s health, the reimplemented tissue was removed three months after delivery, and some fragments re-frozen, thawed and then xenotransplanted into an immunodeficient mouse, with good revascularization and development of pre-antral follicles in the recovered grafts, after four weeks.

Although the analyzed tissue was not reimplanted in human or/and animal models, our preliminary data, obtained in vitro, support the validity of the cryopreservation protocol and suggest the possibility that ovarian tissue might be frozen more than once and re-used in cases where the reimplemented tissue needs to be removed for safety reasons.

5. Conclusions

Removing and re-freezing of the grafted tissue could be a new way of treating not only cancer women with risk of malignant cell recurrence, but also some cases with particu-
lar genetic conditions. Further studies are needed to confirm our outcomes and only the recovery of ovarian function after replanting could provide proof of the true capabilities that the ovarian tissue might be frozen more than once.

Author contributions
MM: conceived and designed the study, analysed all the data, supervised and wrote the manuscript. RP, LDM, RS: performed surgery, revised and edited the manuscript. MM, RF, RV: performed ovarian tissue cryopreservation, analysed all the data. GC: performed histological and immunohistochemical analyses. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The cryopreservation procedure was approved by our local Ethics Committee (S. Orsola-Malpighi Hospital, No. 74/2001/O).

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Conflict of interest
The authors declare no conflict of interest.

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