Ovarian tissue cryopreservation for fertility preservation in 418 girls and adolescents up to 15 years of age facing highly gonadotoxic treatment. Twenty years of experience at a single center

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Abstract
Introduction: The preservation of fertility is an integral part of care of children requiring gonadotoxic treatments for cancer or non-malignant diseases. In France, the cryopreservation of ovarian tissue has been considered and has been offered as a clinical treatment since its inception. The aim of this study is to review 20 years of
activity in fertility preservation by ovarian tissue cryopreservation (OTC) for children and the feasibility of oocyte isolation and cryopreservation from the ovarian tissue at a single center.

**Material and methods:** Retrospective study including patients aged 15 years or younger who underwent OTC, combined for some with oocyte cryopreservation of isolated oocytes, before a highly gonadotoxic treatment for malignant or non-malignant disease was initiated. We describe the evolution of activities in our program for fertility preservation and patient characteristics at the time of OTC and follow up.

**Results:** From April 1998 to December 2018, 418 girls and adolescents younger than 15 years of age underwent OTC, representing 40.5% of all females who have had ovarian tissue cryopreserved at our center. In all, 313 patients had malignant diseases and 105 had benign conditions. Between November 2009 and July 2013, oocytes were isolated and also cryopreserved in 50 cases. The mean age of patients was 6.9 years (range 0.3-15). The most frequent diagnoses in this cohort included neuroblastoma, acute leukemia and hemoglobinopathies; neuroblastoma being the most common diagnosis in very young patients. During follow up, three patients requested the use of their cryopreserved ovarian tissue. All had undergone ovarian tissue transplantation, one for puberty induction and the two others for restoring fertility. So far, no pregnancies have been achieved. Eighty-four patients who had OTC died.

**Conclusions:** Ovarian tissue cryopreservation is the only available technique for preserving fertility of girls. To our knowledge this is the largest series of girls and adolescents younger than 15 years so far reported on procedures of OTC before highly gonadotoxic treatment in a single center.

**KEYWORDS** cancer, children, fertility preservation, gonadotoxic treatment, ovarian tissue cryopreservation

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**1 | INTRODUCTION**

Childhood cancers occurring before the age of 15 years are relatively uncommon diseases. They represent between 0.5% and 4.6% of all cases of cancer. The incidence rate varies from 50 to 200/1 000 000 children worldwide. The current 5 years’ or longer survival rate for children treated for cancer is 80%, which has led to an increasing number of long-term survivors worldwide.

One of the most frequent cancers in childhood is leukemia, representing a third of all childhood cancers. Additional common malignancies at young age are lymphomas and tumors of the central nervous system. Several types of tumors that occur almost exclusively in children are neuroblastoma, nephroblastoma and retinoblastoma.

Childhood cancer treatments consist in variable combinations of surgery, radiotherapy and chemotherapy. These treatments make it possible to obtain a cure in a high proportion of cases but at the price of sequelae including impairment on gonadal function, which seriously alters the quality of life of girls and women, as reproductive health with achievement of motherhood is usually a major wish. Fertility-related information on the impact of a planned treatment and information on fertility preservation should be provided in oncological and hematological care programs, and in cases of gonadotoxic treatments for treatment of benign diseases that may also impair fertility.

Among the various techniques for fertility preservation, cryopreservation of the ovarian tissue is the only method possible in children.

The first series reporting ovarian tissue cryopreservation (OTC) in women was published in 2002 and subsequently several patient series have specifically reported on very young patients, some of them including prepubertal girls (Table 1). Since preservation of ovarian tissue was proposed in 1996, girls of a very young age, between 0.6
and 5 years, have been reported in several series.\textsuperscript{5-7,16} The most frequent types of cancer presented in young patients in those cohorts are leukemias,\textsuperscript{5,8,11,15,16} bone tumors (Ewing’s sarcoma and osteosarcomas),\textsuperscript{5,7,12} Hodgkin lymphoma\textsuperscript{5,17} and neuroblastoma.\textsuperscript{14}

Ovarian tissue cryopreservation may advantageously be coupled with freezing of isolated oocytes either punctured within the visible antrum follicles on the surface of the ovary or collected in the ovary preparation medium when dissecting the ovary.\textsuperscript{17} This additional fertility preservation technique is one way of obtaining isolated mature oocytes in addition to the ovarian tissue without added difficulties and without lengthening the time of patient care.

The purpose of this article is to describe 20 years of experience in fertility preservation through OTC in children up to the age of 15 years, the collection of isolated oocytes during this procedure, the patient characteristics and follow up of the cohort.

2 | MATERIAL AND METHODS

2.1 | Patients

This is a retrospective study including girls and adolescents up to 15 years of age who underwent OTC between April 1998 and December 2018, before a highly gonadotoxic treatment for malignant or non-malignant disease was initiated. From November 2009 to July 2013, we also offered to patients a combination of OTC with freezing of isolated oocytes.\textsuperscript{17} The indication of OTC was established when the treatment planned included conditioning for autologous or allogeneic hematologic stem cell transplantation, with high-dose chemotherapy, in toto abdominal irradiation, pelvic irradiation or ovarectomy. Patients treated for a malignant pathology were all undergoing chemotherapy at the time of OTC. Patients in whom fertility may be prematurely altered, as in case of Turner syndrome, were excluded from this study.

2.2 | Ovarian tissue retrieval

Ovarian tissue retrieval was performed mainly by laparoscopy or minilaparotomy. For patients with neuroblastoma, ovarian retrieval was most often done during laparotomy scheduled for resection of residual tumor.

In the majority of cases, an entire ovary was removed. The ovarian tissue was transferred to the laboratory, in a transport medium that was period-dependent, Leibovitz 15 (Life Technologies, Cergy Pontoise, France) or Brahma 1 (CryoBioSystem, L’Aigle, France), and Ferticult Hepes (Fertipro, JCD, La Mulatière, France), as quickly as possible on ice.

2.3 | Ovarian tissue cryopreservation

As soon as it arrived at the laboratory, the ovary was prepared for freezing. The ovarian cortex was isolated from the medulla and then cut into fragments of 3 x 5 mm with a thickness of 1-2 mm. Each fragment was placed in a cryotube (Nunc, Poly Labo, Strasbourg, France) and then in high security tubes (CryoBioSystem) containing 1 mL of freezing solution composed of the transport medium supplemented with 1.5 mol/L dimethylsulfoxide (Wak-Chemie Medical GmbH, Steinbach, Germany) and 0.1 mol/L sucrose, as cryoprotectant agents, and 10% of the patient’s decompleted and filtered serum or HSA (Vitrolife, Göteborg, Sweden). The slow-freezing protocol has been described previously.\textsuperscript{2} Briefly, after a 30-minute balancing phase at +4°C, the tubes were placed in a programmable freezer. The flow temperature was +4°C. The temperature drop was initially from 2°C/min to −9°C. After automatic or manual seeding, the temperature drop was resumed at a rate of 0.3°C/min to −40°C. The temperature was then dropped from 10°C/min to −140°C. The tubes were finally immersed and stored at the temperature of liquid nitrogen.

2.4 | Collection of immature oocytes

Immature oocytes were recovered both in the follicular fluid collected by aspiration of antral follicles that were visible on the surface of the ovary or in the ovarian preparation medium. The oocytes were then placed in culture medium at 37°C under 5% CO\textsubscript{2} until freezing.

2.5 | Oocyte cryopreservation

The oocytes from the antral follicles and the oocytes found in the ovarian preparation medium were frozen by slow freezing according to the protocol described by Fabbri et al\textsuperscript{18} and then, from 2012, by vitrification (Irvine Scientific, Santa Ana, CA, USA) at the germinal vesicle stage.

2.6 | Studied parameters

Patient characteristics including age, disease and treatment, and the feasibility of oocyte isolation and cryopreservation during the study period were extracted from the medical records. Follow-up data, request for ovarian tissue transplantation during follow up or death were also reviewed.

2.7 | Ethical approval

The study was exempt from institutional review board approval according to French Law No. 2004-800 of 6 August 2004 on Bioethics whereby ovarian tissue cryopreservation is part of patient care. Parents, and patients when it was possible, signed informed consent.

3 | RESULTS

3.1 | OTC activity

Between April 1998 and December 2018, 1031 patients underwent cryopreservation of ovarian tissue before receiving highly gonadotoxic treatment. Among these patients, 418 (40.5%) were girls or adolescents younger than 15 years of age. Pediatric patients were referred from four pediatric hematology departments.
<table>
<thead>
<tr>
<th>Authors (y) [ref]</th>
<th>Patients (n)</th>
<th>Age (y)</th>
<th>Patients 12 y old and under</th>
<th>Patients 15 y old and under</th>
<th>Study period</th>
<th>Most frequent disease</th>
<th>Non-malignant disease</th>
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<td>6.9</td>
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<td>77.7</td>
<td>418</td>
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</table>

AL, acute leukemia; ALL, acute lymphoblastic leukemia; NA, not available.
*Bone tumors include Ewing's sarcoma and osteosarcoma.
(Hôpital Robert Debré, Paris; Hôpital Saint Louis, Paris; Hôpital Necker, Paris; Center intercommunal de Créteil, Créteil) and from two pediatric oncology departments (Institut Gustave Roussy, Villejuif; Institut Curie, Paris).

The proportion of pediatric patients with relation to the total number of cases of OTC at our center has fluctuated from 32% to 125% (Figure 1).

3.2 | Patient age
At the time of OTC, the median age of patients was 6.9 years (0.3-15). In the cohort, 66.5% of patients were younger than 10 years of age (n = 278) and 35.9% younger than 5 years of age (n = 150). Neuroblastoma was more frequently diagnosed in very young patients (median age 3.5 years) and lymphoma in the oldest age groups (median age 13.5 years). The youngest patient undergoing OTC in the cohort was 3.5 months old (Table 2).

3.3 | Diagnosis
Of the 418 patients, 313 had malignant diseases (74.8%) and 105 had non-malignant diseases (25.2%) (Table 2). In the group of children with malignant diseases, 97 had hematological malignancy (23.2%) with leukemia in 78.4% of cases (n = 76) and 218 had a solid tumor (51.7%) with neuroblastoma in 42.7% of cases (n = 93). Hemoglobinopathies were the most common benign disease in the cohort (n = 71; 68.9%).

In the whole cohort, neuroblastoma was the most frequent disease (22.2%) followed by acute leukemia (18.2%) and hemoglobinopathies (17%).

3.4 | Isolated oocyte cryopreservation
Between November 2009 and July 2013, oocyte isolation for cryopreservation was attempted in 124 patients and oocytes obtained from the tissue were cryopreserved in 50 cases (40.3%) (Table 2). Isolated oocytes were cryopreserved in 70.6% of cases of patients with hemoglobinopathies, and in 22.7% of cases of patients with leukemia. The group with the fewest isolated oocytes cryopreserved was the group of patients with solid tumors. The smallest girl for whom it was possible to cryopreserve isolated oocytes was 3.5 months old at the time of OTC and had a non-malignant pathology (porphyria).

3.5 | Patient follow up
During the follow up period, 20.1% (n = 84) of the patients who had OTC died. The group of patients with the highest death rate had malignant solid tumors (26%), of which 28.3% (n = 13) presented with central nervous system tumors, 28% (n = 26) with neuroblastoma, 26.7% (n = 4) with rhabdomyosarcoma and 29.6% (n = 8) with Ewing sarcoma. In the non-malignant group, 31.6% (n = 6) of patients with immunodeficiency died.

3.6 | Requests for transplantation of cryopreserved ovarian tissue
Three patients came back to request transplantation of cryopreserved ovarian tissue. The first request was to restore endocrine function to induce spontaneous puberty. The patient was 10 years old at the time of ovarian storage. The grafting of three fragments of ovarian cortex subcutaneously allowed a spontaneous induction of puberty.19

A second patient requested an ovarian cortex transplant to restore her fertility. She was 12 years old at the time of OTC and had a neuroblastoma. After obtaining the oncologist’s consent, the patient received an ovarian cortex transplant in April 2018. Currently there has been no recovery of ovarian function. A second transplant is scheduled.

The third patient had sickle cell disease and had ovarian conservation at the age of 11.2 years. She wishes to have a child. An ovarian cortex transplant was performed in February 2019.

4 | DISCUSSION
To our knowledge, this is the largest series of young patients including girls and adolescents younger than 15 years of age that

![FIGURE 1](wileyonlinelibrary.com)
underwent fertility preservation through OTC at one center. Additionally, the isolation of oocytes during tissue preparation for OTC in girls was performed in a smaller series of patients. Our pediatric patient cohort is the largest so far reported undergoing OTC for fertility preservation. The median age of the patients in our cohort is also below that reported by other authors, usually >10 years; the median age in our previous published series in 2002 was 5 years and in our current series is 6.9 years. The percentage of patients aged 0-12 years varies according to the series from 16% to 87%; it is 77.7% in our current series. Taking into account the study period of each publication and the average number of girls and adolescents younger than 15 years of age, the rate per year varies approximately from 1.19 to 20.4.

The patients in our study had a high proportion of pediatric-specific diseases such as neuroblastoma but also common childhood diseases such as leukemia and central nervous system tumors. Apart from leukemia, the most common pathologies found were bone tumors5-7,12 and lymphoma4-8,11,13 which we did not find in our series. A high number of our patients had hemoglobinopathy, in particular sickle cell disease. This is linked to our collaboration with the expert center for sickle cell disorders at the Center Hospitalier Intercommunal de Créteil,20 which offers a curative treatment of sickle cell disorders by Hematological Stem Cell Transplantation (HSCT) with a myeloablative conditioning regimen known to be highly gonadotoxic.21 During OTC, it is possible to freeze isolated oocytes. In 2003, Revel et al17 described for the first time oocyte isolation in children younger than 12 years with seven, eight and seven oocytes isolated from the ovarian cortex of patients aged 5, 8 and 10 years, respectively. This is important in certain pathologies at risk of localization of the disease in the ovary that will preclude the ovarian tissue transplantation, such as in leukemia. In our series, isolated oocytes were found only in 27.3% of cases. Having isolated oocytes may increase future fertility restoration possibilities of these patients.

In our series, 20.1% of the patients died. This rate is similar to that reported by other teams4,5,13 but higher than the series published by Jadoul et al10 (13.8%). To date, three patients of our series had requested ovarian tissue transplantation. They were all prepubertal at the time of OTC. If we take into account only living patients who are currently 18 years of age or older, the utilization rate is 2.2% (3/149) of the population. This utilization rate is lower than that estimated by Jadoul et al22 which was 3.9%, but those patients had an average age of 22.3 ± 8.8 years at the time of OTC.

| TABLE 2 | Patient series of 418 girls and adolescents younger than 15 y of age who underwent ovarian tissue cryopreservation between April 1998 and December 2018 |
|--------------------------|--------------------------|--------------------------|--------------------------|
| Age at OTC | Number of OTC | Oocytes for cryopreservation/OTC* | Deceased patients |
| Median [range] | n (%) | n (%) | n (%) |
| Malignant hematological diseases | | | |
| Acute leukemia | 7.2 [6-14.9] | 76 (18.2) | 5/22 (22.7) | 15 (19.7) |
| Lymphoma | 13.5 [4.6-14.8] | 10 (2.4) | 1/6 (16.7) | 2 (20.0) |
| Others | 12.5 [1.1-14.4] | 11 (2.6) | 3/5 (60.0) | 1 (9.0) |
| Total | 8.4 [6-15.0] | 97 (23.2) | 9/33 (27.3) | 18 (18.6) |
| Solid malignant tumours | | | |
| CNS tumors | 7.6 [1.6-13.7] | 46 (11.0) | 5/13 (38.5) | 13 (28.3) |
| Neuroblastoma | 3.5 [5.4-14.8] | 93 (22.2) | 3/20 (15.0) | 26 (28.0) |
| Rhabdomyosarcoma | 4.5 [1.3-14.3] | 15 (3.6) | 0/2 (0.0) | 4 (26.7) |
| Ewing sarcoma | 12 [4.8-15] | 27 (6.5) | 1/8 (12.5) | 8 (29.6) |
| Nephroblastoma | 4.5 [3.2-12] | 13 (3.1) | 1/2 (50.0) | 2 (15.4) |
| Others | 12.7 [1.8-15] | 22 (5.3) | 1/4 (25.0) | 3 (13.6) |
| Total | 5.2 [5-15] | 216 (51.7) | 11/49 (22.4) | 56 (26.0) |
| Non-malignant diseases | | | |
| Hemoglobinopathies | 8.4 [2.1-14.9] | 71 (17.0) | 24/34 (70.6) | 2 (2.8) |
| Immune deficiency | 5.3 [1.6-14.5] | 19 (4.5) | 5/10 (50.0) | 6 (31.6) |
| Aplastic anemia | 8.8 [1.6-14.3] | 10 (2.4) | 0/0 (0.0) | 2 (25.0) |
| Others | 12.1 [3-14.1] | 5 (1.2) | 1/1 (100) | 0 (0.0) |
| Total | 8.3 [3-14.9] | 105 (25.1) | 30/42 (71.4) | 10 (9.5) |
| TOTAL | 6.9 [3-15] | 418 (100) | 50/124 (40.3) | 84 (20.1) |

*Between November 2009 and July 2013, oocytes were also isolated and cryopreserved for 50 of the patients. CNS, central nervous system; OTC, ovarian tissue cryopreservation.
To date, transplantation of cryopreserved ovarian tissue has resulted in births of at least 130 children but data on transplantation of ovarian tissue removed before puberty are scarce. Currently in the literature, four patients under 15 years of age at the time of OTC have used their cryopreserved ovarian tissue. The first publication showing the functionality of ovarian tissue cryopreserved before puberty was published in 2012. This was a patient with sickle cell disease in whom ovarian freezing was carried out at age 10 before a myeloablative conditioning regimen followed by allogeneic HSCT. Twenty-seven months after the HSCT, the patient and her mother returned to request an ovarian tissue autotransplantation to induce spontaneous puberty because she had premature ovarian failure. A heterotopic autotransplantation of three fragments of ovarian cortex succeeding in inducing puberty with the onset of the first menstrual period 8 months after the ovarian transplantation. The following year, Ernst et al confirmed that ovarian cortex transplantation could induce puberty in a patient who was 9 years old at the time of OTC prior to treatment for Ewing’s sarcoma.

The first birth obtained after transplantation of ovarian tissue cryopreserved before menarche was reported in 2015. This patient underwent OTC before HSCT for the treatment of sickle cell disease. At the time of the ovarian cryopreservation she was almost 14 years old and had not had any periods yet. Ten years later, the patient had a premature ovarian failure and requested an ovarian tissue transplantation to restore her fertility. She had her first menstrual period 5 months after the ovarian transplantation and became spontaneously pregnant more than 2 years after the transplantation, giving birth to a healthy boy.

The youngest patient at the time of ovarian freezing, who gave birth after ovarian cortex transplantation, was 9 years old at the time of tissue retrieval. She had beta-thalassemia treated by HSCT preceded by gonadotoxic conditioning regimen before HSCT.

These rare publications confirm that beyond the age of 9, ovarian tissue can be functional after transplantation. It remains to be determined whether this can be applied for girls younger than 9 years old.

5 | CONCLUSION

The field of fertility preservation is constantly evolving. There is an ethical obligation of clinicians to provide information on impact of cancer treatment on future fertility and to discuss fertility issues with prepubertal cancer patients and their parents, in order to preserve the chances of future parenthood. For girls who require a highly gonadotoxic treatment, OTC is the only technique that can be offered. The results of the previously cryopreserved OTC are encouraging. It is still necessary to wait a few more years for more results, especially in patients younger than 9 years at the time of OTC. Our series shows that OTC can be proposed in a wide range of diseases all requiring a highly gonadotoxic treatment.

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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