Mean age at primary diagnosis was 12.1 years (range: 7–17.2 years) while mean age at the time of the procedure was 15 years old (range 7–17.9 years), and 10 patients were pre-pubertal at the time of surgery. Patients were affected by: lymphoma (15), osteosarcoma/Ewing sarcoma (7), rhabdomyosarcoma/synovial sarcoma (5), neuroblastic tumor (3), medullary aplasia (2), Chronic granulomatous disease (2), ovarian teratoma (1), medulloblastoma (1).

Main indications for cryopreservation were chemotherapy and radiotherapy (27), chemotherapy for bone marrow transplantation (5), chemotherapy (4), removal of both ovaries for neoplastic risk (2).

The mean operative time was 55 min. Only one procedure was performed in open surgery in a case with bilateral ovarian teratoma. Drainage was not necessary in any of the procedures and patients were discharged on the second post-operative day. In 5 cases the sampling was bilateral. The right ovary was sampled in 28 cases, while the left ovary was selected in the remaining 5 patients.

3.4 Center 4

In Center 4, the program of LOTC in girls and adolescents with onco-hematologic diseases started in January 2020. A total of 18 procedures were performed up to December 2022. Patients were diagnosed with leukaemia (4), non-Hodgkin's lymphoma (3), Hodgkin's lymphoma (8), acquired bone marrow aplasia (1), botryoid vaginal rhabdomyosarcoma (1) and Ewing's sarcoma of the pubis (1). Ovarian preservation was performed prior to conditioning for bone marrow transplantation or initiation of gonadotoxic chemotherapy. Age at diagnosis and at intervention varied widely. Patients with leukaemia were diagnosed at birth and preservation surgery was performed on average at 4.86 years (2–10 years). The mean age at diagnosis for the remaining patients was 13.7 years (except for a patient with vaginal rhabdomyosarcoma who was diagnosed at 15 months) and, in this group, surgery was performed at an average of 2.68 months after diagnosis. Only 3 patients were pubertal at the time of surgery.

Depending on the age of each patient and the intraoperative findings (ovarian volume and macroscopic characteristics), it was decided which ovary was sampled and whether to perform an ovarian cortex removal (5) or a total oophorectomy (11), always sampling only one ovary. No bilateral ovarian cortex removal was performed. Seventy-five percent of the procedures were performed on the right ovary. All procedures were approached laparoscopically and none required conversion. Mean operative time was 65 min.

One patient subjected to unilateral ovarian cortex excision required urgent re-laparoscopy due to hemoperitoneum and sudden anemization. Hemoglobin check was not routinely carried out. Patients were discharged from surgery on the same day or the day after the operation. Bone marrow transplantation or chemotherapy was initiated at a mean of 14.7 days after surgery.

No malignant cells were identified at the histopathology evaluation.

4 Discussion

This study reports the surgical outcomes of a large multicentric series of young patients including girls and adolescents younger than 17 years of age at diagnosis, who underwent fertility preservation through LOTC at four European high-volume Pediatric Centers.

The population in this 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 commonly observed pathologies were sarcomas and lymphomas.

Regarding the approach to surgery, there are some differences between Centers.

Bilateral harvesting in Center 1 is opposed to unilateral biopsy in Center 2, 3, and 4, adding the pre-operative ultrasound evaluation.

The drainage was routinely left in place only in Center 2. No minor or major complications were reported in Center 2 and 3, while a postoperative bleeding that needed a re-laparoscopic exploration and a coagulation of the ovarian surface was registered both in Center 1 and 4.

Different techniques for LOTC are reported in literature, such as ovarian cortical biopsies, partial or unilateral total oophorectomy. Some Centers opted for total unilateral ovarian removal, because of the theoretical reduced risk of bleeding and ovarian damage; furthermore, prepuberal ovaries are smaller than their puberal counterpart, so the chances of collecting a sufficient amount of ovarian tissue is greater with a wider removal (2). On the other hand, some patients do not suffer from POF, but rather have a low ovarian reserve and the withdrawal of the entire organ could exacerbate the failure. Moreover, for the patient, it is psychologically easier to accept multiple ovarian sampling than a total oophorectomy (6).

Regardless of the surgical procedure preferred, the laparoscopic approach is the most common in children and adolescents. However, the minimal invasive technique can increase the risk of post operative bleeding and anemia. There is no universal consensus on which is the optimal and safest hemostatic procedure. Electrocauterization is arguably the fastest and most secure way; at the same time, it has been reported that repeated cauterization could cause an important reduction in the ovarian reserve, due to irreversible vessel damage and the subsequent tissue reaction. The intracorporeal suture of the ovarian bed is undoubtedly the best option in terms of preservation of the remaining ovarian tissue; nevertheless, this is difficult to accomplish in a short time and it requires a long hemostatic time, so is unsuitable for emergency procedure (7).

The three most common techniques for ovarian tissue cryopreservation are the following: vitrification, slow-freezing, and ultra-rapid freezing (8). The slow-freezing technique, used for the ovarian tissue cryopreservation of this series of patients, was firstly described by Behrman and Savada in 1966 (9–11). This technique consists of a gradual cooling of the sample using liquid nitrogen and aims to reduce ovarian toxicity related to a lower concentration of the cryoprotectants used. The

cryoprotectant is added by sequential dilution in two steps, with minor modifications. The ovarian tissue fragments are incubated at low temperatures between 2 and 10 degrees centigrade.

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, 4 patients under 15 years of age at the time of OTC have used their cryopreserved ovarian tissue (12).

LOTC was firstly described by Hovatta et al. in 1996 (13). However, the first publication showing the functionality of ovarian tissue cryopreserved before puberty was published in 2012. It showed 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 HSCT, the patient and her mother returned to request an ovarian tissue autotransplantation to induce spontaneous puberty because she had POF. A heterotopic autotransplantation of three fragments of ovarian cortex succeeded in inducing puberty with the onset of the first menstrual period 8 months after the ovarian transplantation (14).

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 (15).

The first birth obtained after transplantation of ovarian tissue cryopreserved before menarche was reported in 2015 (16). This patient underwent OTC before HSCT for the treatment of sickle cell disease. At the time of the ovarian cryopreservation, she was 14 years old and she had still not had a menstrual period. Ten years later, the patient had a POF and requested an ovarian tissue transplantation to restore her fertility. She had her first menstrual period 5 months after the procedure 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 a gonadotoxic conditioning regimen before HSCT (17).

These rare publications confirm that beyond the age of 9, ovarian tissue can be functional after transplantation (4). It remains to be determined whether this can be applied for girls younger than 9 years old.

The main limitation of the present study is its focus only on the short-term surgical outcomes, examining the incidence of short-term postoperative complications in the first thirty days after surgery and in the following six months, without examining the outcomes in terms of endocrine function and fertility of our patients. Future studies related to these variables will be necessary to understand the global impact of LOTC on the endocrine and fertility outcomes of oncological patients.

Another limitation of the present study is represented by the heterogeneity of the laparoscopic technical aspects of each Centers, such as the diameter of the trocar used for the first access and the time frame in which the data were collected (2 years for Center4, 11 years for Center3, 20 years for Center1 and 2). Moreover, the global data collection of patients submitted to surgery over a period of approximately 20 years could be burdened by biases linked to technical and technological improvements in minimally invasive surgery, although both the dimensions of the trocars and the type of surgical instruments used for LOTC have been standardized in each Center for the present series of patients. Due to the young age of the patients involved, the routine use of a 5 mm optic trocar for the first access should be considered in further studies.

Regarding ethical and medico-legal issues, a fertility preservation technique should ideally be evidence-based, should not create false hopes, unrealistic expectations or harm the patient.

The problem with cryopreservation is the experimental label which has not yet been removed in many countries, especially because the evaluation of effectiveness takes many years and because of the risk of reimplantation of cancer cells. It is well known, for patients with acute lymphoblastic leukemia or lymphoma, that the ovarian stroma may contain malignant cells. Rosendahl et al. reported 26 cases of ovarian tissue taken from woman with diagnosis of leukemia and, even if immunohistochemistry was unable to detect malignant cells in the cortex, in most of the sampling, it was possible to discover potential tumoral cells using PCR (18).

Although, up to date, there is no evidence that these cells are viable or that the disease could relapse once they are reimplanted, in these cases the procedure is not recommended. There are now numerous studies about alternative methods for fertility restoration in patients with residual malignant cells in the stroma, such as follicle isolation, *in vitro* follicle maturation or *in vitro* fertilization.

If these factors can create some doubts, it is necessary to consider that many studies attest distress, anxiety and depression related to future infertility. Cryopreservation is the only way for pediatric surgeons to preserve young patients' fertility in prepubertal age. We must also consider the positive effect that thinking about the future provides for patients dealing with an oncologic pathology.

As for intra- or post-operative complications the rate remains low in most of the studies.

Beckmann et al., demonstrated that the rate of complications for ovarian tissue removal and transplantation is so low that it can be compared to those from standard laparoscopy (19).

Furthermore, laparoscopy offers a great advantage in young cancer patients as it allows a direct oncological staging thanks to the exploration of the entire abdomen. Finally, the advantage of the pediatric age is precisely the question of time, that is the fact that we do not know how much progress science will have made when this tissue will be requested by patients in 10 or 20 years, when new therapeutic possibilities might be available.

Within a few years, long-term data regarding this cohort of patients will be available, which will add to the short-term surgical data, already presented in the present study, information about the impact of the LOTC on long-term fertility and endocrine outcomes.

5 Conclusions

LOTC is a promising modality to preserve fertility in pediatric population submitted for gonadotoxic therapies. Even if there are different techniques and approaches, the procedure is safe, easy to perform and reliable, and offers a minimal invasive approach in high-risk patients, without delaying the onset of life saving chemo or radiotherapy. This series of 311 patients from different European Centers was comparable in terms of technical approaches and outcomes. A team of specialized surgeons is needed to grant and develop a standardized procedure. Future studies including endocrine and fertility outcomes will clarify the global and long-term clinical significance of LOTC.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Comitato Etico Area Vasta Emilia Centro (AVEC). CET Interaziendale AOU Città della Salute e della Scienza di Torino. Comitato Etico Ospedale San Martino. Comité de Ética de la Investigación con Medicamentos (CEIm). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

Author contributions

FP: Supervision, Writing – original draft. GF: Visualization, Writing – original draft. LL: Methodology, Writing – original draft. TG: Methodology, Writing – review & editing. MM: Methodology, Writing – review & editing. SA: Methodology, Writing – review & editing. FP: Methodology, Writing – review & editing. MB: Visualization, Writing – original draft. AD: Visualization, Writing – original draft. CG: Visualization, Writing – original draft. GP: Visualization, Writing – original draft. GM: Methodology, Writing – review & editing. FG: Methodology, Writing – original draft. ML: Methodology, Writing – review & editing. RG: Conceptualization, Supervision, Writing – original draft.

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Conflict of interest

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