UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE IN MEDICINA MOLECOLARE

CURRICULUM IN MEDICINA CLINICA E SPERIMENTALE

Improving fertility preservation strategies in women with cancer: development of a murine model of gonadotoxicity and clinical prognostic signatures of gonadal damage

DoS: Dr.ssa Giorgia Mangili

Second Supervisor: Prof. Alberto Revelli

Tesi di DOTTORATO di RICERCA di Raffaella Cioffi

matr.015643

Ciclo di dottorato XXXV°

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CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

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Data /Date 18/11/2022 Firma /Signature .....

DECLARATION

This thesis has been:

- composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.
- has been written according to the editing guidelines approved by the University.

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All the results presented here were obtained by myself, except for:

1) Mice CEUS experiments (Results, chapter 5.1) were performed in collaboration with dr. Laura Perani, Division of Experimental Imaging, San Raffaele Scientific Institute, Milan, Italy.
2) Immunohistochemical analyses (Results, chapter 5.1) were performed by dr. Antonella Monno, Division of Immunology, Transplantation, and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy.

The following data have been published in peer-reviewed journals during the second and third year of the PhD program:

1) Studies on human ovarian tissue (Results, chapter 5.4.1) (Cioffi, R., Cervini, L., Taccagni, G., Papaleo, E., Pagliardini, L., Bergamini, A., Ferrari, S., Mangili, G., & Candiani, M. (2022). A prospective, observational study of chemotherapy-induced ovarian damage on follicular reserve and maturation. Archives of Gynecology and Obstetrics, 306(5), 1723–1729. https://doi.org/10.1007/s00404-022-06692-0)
2) Clinical studies (Results, chapter 5.5.1; chapter 5.5.3) (Cioffi, R., Fais, M. L., Bergamini, A., Vanni, V. S., Pagliardini, L., Papaleo, E., Mangili, G., & Candiani, M. (2022). Ovarian failure risk in post-pubertal patients with cancer: A prognostic model. Future Oncology, 18(19), 2391–2400. https://doi.org/10.2217/fon-2022-0078; Cioffi, R., Mangili, G., Sarais, V., Cervini, L., Longo, V., Bergamini, A., Stella Vanni, V., Pagliardini, L., Candiani, M., & Papaleo, E. (2021). Do stage and grade of malignancy impact fertility

preservation in breast cancer patients? Journal of Gynecology Obstetrics and Human Reproduction, 50(10), 102215. https://doi.org/10.1016/j.jogoh.2021.102215).

All sources of information are acknowledged by means of reference.

Abstract

Background: Chemotherapy is known to determine an impairment of fertility through the depletion of ovarian reserve. Current fertility preservation strategies in women with cancer include GnRH-a administration during chemotherapy, oocyte cryopreservation and ovarian tissue cryopreservation (OTC). The effects of GnRH-a administration during chemotherapy have been studied both in pre-clinical and clinical settings, with conflicting evidence. Oocyte cryopreservation is nowadays an established technique, even though women with different diagnoses of cancer can display mixed response to ovarian stimulation. OTC is emerging as the ultimate strategy for fertility preservation; however, it is unclear whether it has a potential in women who already started cancer treatment out of clinical necessity. Besides gonadotoxicity, a new emerging field of research is the impact of cancer treatments on the uterus, currently unknown.
A deeper knowledge of these aspects would be required in order to provide women with the best opportunity for fertility preservation.
Methods: This project included the development of a murine model for the study of gonadotoxicity through a platform of high-resolution Contrast-Enhanced Ultrasound. Mice were administered two different chemotherapeutics (doxorubicin and cyclophosphamide) and the effects of these drugs on follicular development and perfusion were studied in vivo, with and without concurrent GnRH-a administration. In parallel, histological follicular counts of human ovarian tissue biopsies of patients undergoing OTC before and after the beginning of chemotherapy were compared. The project also developed through the analysis of clinical data from a dataset of 348 patients receiving counselling for fertility preservation at our Unit, to identify factors associated with impaired fertility and premature ovarian failure (POF), as well as poorer responses to controlled ovarian stimulation (COS). Finally, a prospective ultrasound-based protocol was developed for the study of uterine morphology and perfusion after chemotherapy in oncological patients.
Results: Our murine experiments showed that GnRH-a treatment in mice did not inhibit folliculogenesis at 14 days after administration. However, it determined a decrease of ovarian perfusion, confirmed by a reduction of CD31 staining within ovarian tissue. Mice treated with cyclophosphamide had a significant increase of peak enhancement and wash-in rate, while co-administration of GnRH-a reduced this effect. Mice treated with doxorubicin showed an increase of mean transit time. Adding decapeptyl to doxorubicin did not significantly alter perfusion parameters. Follicular counts on human ovarian tissue biopsies confirmed a comparable number of primordial follicles between chemotherapy-treated and chemotherapy-naïve biopsies. The analysis of COS outcomes in our breast cancer patients cohort showed the same number of retrieved mature oocytes in high-stage and low-stage cancers, as well as high-grade and low-grade cancers, however women with high-grade breast cancer showed lower antral follicular counts and required higher doses of gonadotropins during stimulation. Patients diagnosed with a sarcoma did not show worse COS outcomes compared with a cohort of controls without a cancer diagnosis. Next, a prognostic model to assess the probability of POF in young women with cancer was developed from a regression analysis performed on our dataset. Finally, the ultrasound protocol for the study chemotherapy-related uterine changes was developed and the first preliminary results generated the hypothesis that chemotherapy could determine an increase of uterine artery resistance.
Conclusions: Currently, the best outcomes for fertility preservation can only result by an individualized approach and a combination of different strategies, before or right after the beginning of treatments. Gonadal protection could be different depending on each drug used. The damage could not be limited to the gonads, but also involve the uterus: further studies are urgently needed to develop protective strategies on this front. At the current state of knowledge, GnRH may be offered in addition, and not instead of other strategies.

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1. Acronyms and abbreviations

POF: premature ovarian failure

POI: premature ovarian insufficiency

AMH: anti-Mullerian hormone

HPG: hypothalamic-pituitary-gonadal

OTC: ovarian tissue cryopreservation

COS: controlled ovarian stimulation

OHSS: ovarian hyperstimulation syndrome

GnRH-a: gonadotropin-releasing hormone agonists

hCG: human chorionic gonadotropin

AI: aromatase inhibitor

IVF: in-vitro fertilization

ART: assisted reproduction techniques

IVM: in-vitro maturation

FSH: follicle-stimulating hormone

LH: luteinizing hormone

RCT: randomized controlled trial

CEUS: contrast-enhanced ultrasound

PE: Peak Enhancement

WiR: Wash-in rate

WiAUC: Wash-in Area Under the Curve

WiPi: Wash-in Perfusion index

AUC: Area under the curve

PI: Perfusion index

mTT: mean Transit Time

AFC: antral follicular count

MII: metaphase II

VAI/AI: Vincristine, Adriamycin and Iphosphamide/Adriamycin and Iphosphamide

ABVD: Adriamycin, Bleomycin, Vinblastine and Doxorubicin

2. List of figures and tables

3. Introduction

The introduction of chemotherapy in the treatment of cancer was one of the greatest developments of modern medicine, allowing a significant improvement of patients' life expectancy. It is a fact that death rates have been declining consistently over the course of the years for many types of cancer (American Cancer Society, 2019).

This remarkable result entails the need to address clinical issues that are specific to the population of young cancer survivors, who are expected to live for many years after disease remission. Aspects related to quality of life of cancer survivors have become crucial in recent years.

One of the most important issues in the population of young women surviving cancer is the risk of post-treatment infertility and premature ovarian failure (POF). It is estimated that, as a result of gonadotoxic treatments, at least one third of the female cancer population undergoes premature ovarian insufficiency (POI) (Bedoschi et al., 2016).

Many types of chemotherapy and pelvic radiotherapy are known to be gonadotoxic, with different underlying mechanisms being hypothesized (Bedoschi et al., 2016).

As acknowledged by clinicians, most cancer survivors express reproductive concerns, and many oncological patients have a strong desire to have children after the cancer is cured (Ruddy et al., 2014).

Having a pregnancy after cancer does not imply an increased risk of cancer recurrence, even in the case of hormone-dependent tumors (Lambertini et al., 2018)(Weibull et al., 2016). Yet, pregnancy rates are lower in the cancer survivors' population compared to unaffected women (Jayasinghe et al., 2018).

Infertility is generally a well-known source of emotional distress for patients, but also premature menopause is an issue, due to its dramatic impact on health and quality of life.

Menopausal symptoms such as hot flashes, insomnia, dyspareunia, mood disturbances can be very debilitating. Patients often experience changes in their sexuality with detrimental consequences on their relationships. Furthermore, the effects of long-term estrogen deprivation, such as osteoporosis, cardiovascular disorders and neurocognitive decline compromise health and aging (Hickman et al., 2016).

The most recent guidelines recommend that oncologists address the reproductive concerns as soon as possible with their patients, ideally before the beginning of cancer

treatment (Lambertini et al., 2020). Patients diagnosed with cancer should be counselled about their risk of subsequent infertility and, if considered safe from an oncological perspective, be referred to a fertility clinic for access to fertility preservation strategies. Therefore, management of reproductive-aged women with a malignancy always requires a multidisciplinary team to take into account treatments' potential gonadotoxicity, patients' age and ovarian reserve, and risks/benefits of each fertility preservation strategy.

The individual risk of infertility results from a combination of patient-related and treatment-related factors and is not easily determined upfront.

3.1 Mechanisms of gonadotoxicity

In the human ovary, there is a finite number of oocytes, deriving from the primordial follicles pool. Throughout the entire life, primordial follicles are cyclically recruited and activated, with resumption of meiosis in a small fraction of them. The majority of primordial follicles undergoes atresia during development.

So, the loss of primordial follicles, which constitute the ovarian reserve, is continuous throughout a woman's life until depletion, that happens right before menopause.

While it is true that some chemotherapeutic compounds are more gonadotoxic than others, the entity of the primordial follicle pool at the time of treatment is also very important in determining the risk of acute ovarian failure. An acute POF is more likely to occur in older women because their primordial follicle pool is already reduced. Younger women tend to experience instead an anticipated menopause compared with unaffected women (Morgan et al., 2012).

The temporary amenorrhea that many women experience during and immediately after treatment is the result of the acute destruction of the growing follicle pool. Damage to the resting, primordial follicle population, is the mechanism leading to POF. In most cases, the destruction of this pool is limited, and this allows resumption of menses for a variable number of years; in some cases, especially when very high doses are administered, the reduction is nearly complete and POF manifests itself shortly after treatment. This phenomenon is called "two-hit effect" (Morgan et al., 2012; Petrek et al., 2006).

The loss of primordial follicles might be the result of a direct damage, but also, given their quiescence, it is more likely an indirect consequence of the acute loss of activated follicles. In fact, most chemotherapeutic agents only hit mitotically-active and meiotically-active cells.

The following mechanisms of gonadotoxicity are involved in chemotherapy-mediated ovarian damage:

1) direct DNA damage (double-strand breaks and inter-strand crosslinking) to growing follicles (Rosendahl et al., 2010): the immediate consequence of the loss of dividing follicles is amenorrhea that occurs during treatment; alkylating agents and alkylating-like platinum complexes (i.e. cisplatin and anthracyclines) are known to cause double-strand breaks and consequent apoptosis;

2) follicular "burnout": the demise of growing follicles and consequent local reduction in anti-Mullerian hormone (AMH) concentrations causes an upregulation in the PI3K/PTEN/Akt signalling pathway and the Hippo pathway and loss of suppression of activation in primordial follicles (Roness et al., 2013); as a result, more follicles leave the resting pool and start developing, undergoing atresia shortly after;

3) stromal damage, with fibrosis and hyalinization of small vessels, which lead to ischemic damage;

4) damage to follicular support cells, especially granulosa cells, with indirect damage to oocytes (Morgan et al., 2012). In particular, apoptosis of granulosa cells induces a decrease in estrogen levels that can remove the negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis, further contributing to the increased follicular recruitment.

Figure 1 summarizes the mechanisms that are hypothesized in chemotherapy-mediated ovarian damage.

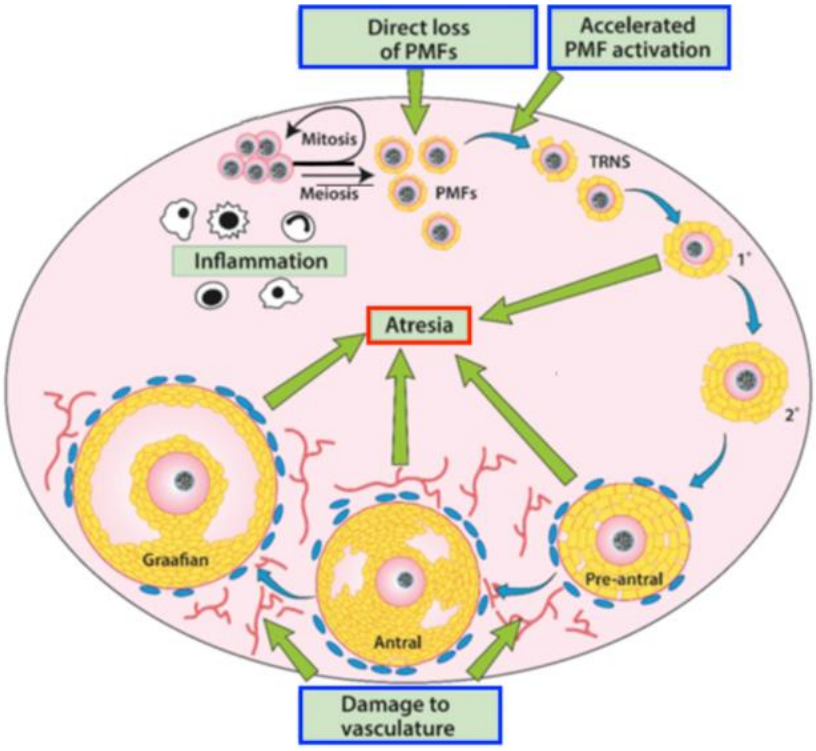

Figure 1. *Main mechanisms through which chemotherapy damages the ovary (modified from Spears et al., 2019. According to "fair use principle", for educational purposes only.)*

Below are reported the principal mechanisms implied in the gonadotoxicity of the most commonly used chemotherapeutic drugs in premenopausal women.

3.1.1 Cyclophosphamide

This alkylating agent is considered among the most gonadotoxic drugs. It directly binds to DNA causing double-strand breaks, irrespective of cell cycle phase. It also activates the apoptotic cascade through several mechanisms. It is possible that it damages both resting and growing follicles, as well as somatic cells (Morgan et al., 2012).

Studies have shown that cyclophosphamide administration causes a reduction in the primordial follicle pool that is proportional to its dose (Meirow et al., 1999).

3.1.2 Cisplatin

Like alkylating agents, it binds to DNA interfering with cell replication. It may preferentially target oocytes, but data is scarce (Gonfloni et al., 2009).

3.1.3 Doxorubicin

It belongs to the anthracycline family. Its mechanisms of action include an inhibition of DNA transcription and replication and mitochondrial damage, which ultimately lead to cellular apoptosis. It is likely that the gonadotoxicity of doxorubicin depends on damage to somatic cells more than a direct damage to oocytes (Morgan et al., 2012).

3.2 Chemotherapy and uterine damage

While the impact of chemotherapy and radiotherapy on gonadal function has been extensively studied, the effects of cancer therapies on the uterus mostly remain unknown (Griffiths et al., 2020). As a consequence, currently all fertility preservation strategies focus on ovarian protection, while there are no protective approaches for the uterus and endometrium, which would be essential for the achievement of a healthy pregnancy.

Most of the available evidence on uterine damage focuses on the exposure to pelvic radiation (Reulen et al., 2009), that has been associated with low birth weight, prematurity, fetal malposition, placental disorders and uterine rupture (van de Loo et al., 2019). Radiation injury to the uterus is mediated by the formation of free radicals that can damage DNA and proteins: this leads, in a dose-dependent manner, to decreased uterine volume and blood flow, myometrial fibrosis and impaired endometrial response to sex steroids (Critchley et al., 2002)(Teh et al., 2014). Some studies reported reduced uterine volume, absent blood supply, and reduced endometrial thickness after total body irradiation (Larsen et al., 2004).

Conversely, very little is known about the effects of uterine exposure to chemotherapy. Pregnancy rates are lower in women that have been exposed to chemotherapy (Garg et al., 2020). However, in most studies evaluating fertility outcomes, it remains very difficult to isolate uterine effects from ovarian contribution. In a recent study, patients exposed to chemotherapy (without pelvic radiation) showed a higher prevalence of

preterm birth and small for gestational age compared to non-cancer controls (Anderson et al., 2017). It is unlikely that these effects can be attributed exclusively to ovarian impairment.

The exact molecular mechanisms of chemotherapy-mediated uterine damage are not yet characterized. Alkylating agents, chemotherapeutics commonly used in reproductive age, can generate free radicals damaging DNA, much like radiation; also other chemotherapeutics can damage DNA or disrupt cell cycle, potentially damaging myometrial and endometrial cells (Garg et al., 2020).

3.3 Fertility preservation strategies

Most fertility preservation strategies are indirect solutions to the problem of gonadotoxicity of chemotherapeutic agents, as they aim at creating an external banking of gametes to resort to whenever fertility seems to be impaired. The suggested age cut-off for embryo/oocyte cryopreservation is 40 years, while for ovarian tissue cryopreservation (OTC) some studies indicate a lower cut-off, around 36 years (Lambertini et al., 2020).

Figure 2 summarizes available fertility preservation strategies in oncological patients.

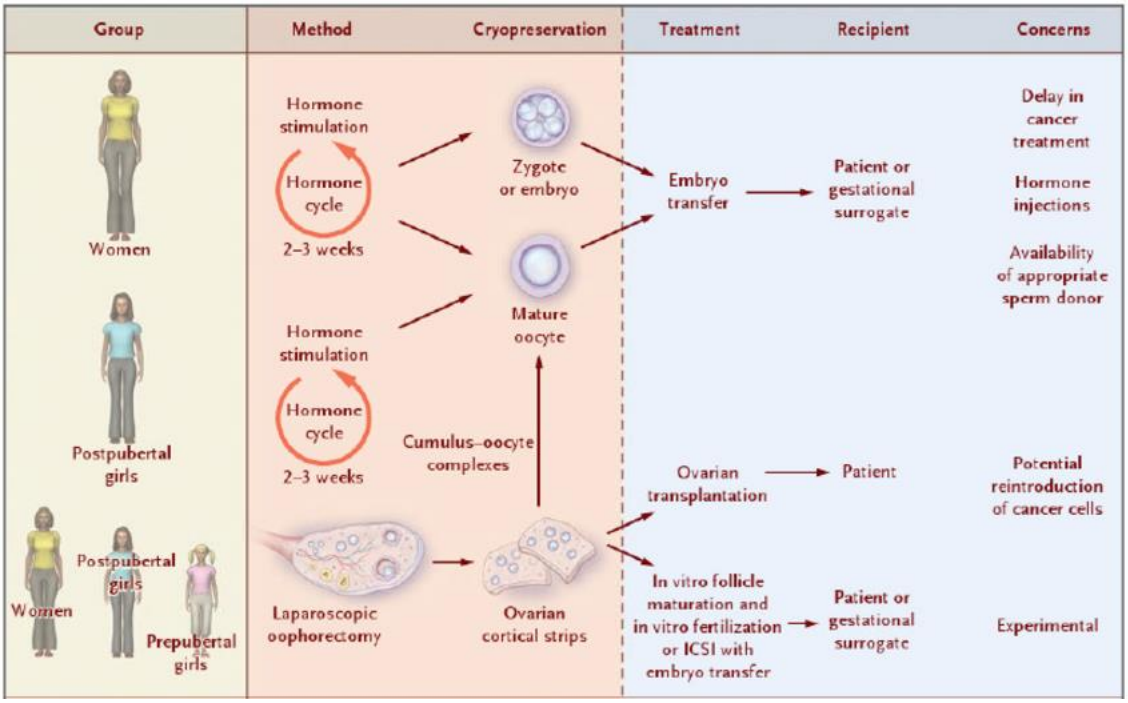

Figure 2. *Available fertility preservation strategies in pre-pubertal and post-pubertal patients with cancer (from Jeruss & Woodruff, 2009, according to "fair use principle", for educational purposes only)*

3.3.1 Embryo cryopreservation and Oocyte cryopreservation

Embryo cryopreservation is an established technique in post-pubertal women with a committed partner. Transfers of cryopreserved embryos lead to the same live birth rates as fresh embryo transfers (around 43% per transfer in women younger than 35) (Oktay et al., 2015). As with other reproductive assisted techniques, live birth rates decline as patients age.

However, embryo cryopreservation is an option that can be rarely applicable in the setting of fertility preservation in oncological patients, due to the need for the presence of a male partner at diagnosis (unsuitable in younger patients) and the ethical and legal consequences of embryo disposition in case the partner is no longer available, or the patient dies of disease. Additionally, both partners must sign the consent for embryo

utilization, thus a divorced woman might encounter obstacles in accessing her previously cryopreserved embryos.

Oocyte cryopreservation is considered the gold standard approach for fertility preservation in post-pubertal patients, since it can be offered to patients without a partner.

The introduction of vitrification allowed a significant technical improvement in this procedure compared to the previously used technique of slow-freezing. The live birth rate with vitrification per oocyte thawed is 6.5%, and the cumulative birth rates increase with the number of cryopreserved oocytes (Cobo et al., 2015).

Both techniques require controlled ovarian stimulation (COS) for approximately 2 weeks. Stimulation after the beginning of chemotherapy is not recommended, since there may be concerns associated with genetic impairment of retrieved oocytes (Ngu & Ngan, 2016). Due to the potential genetic damage to oocytes, COS can only be offered to patients whose treatment can be safely delayed. For patients carrying a specific mutation, such as Breast-related Cancer Antigen (BRCA) 1-2, preimplantation genetic testing might prevent passing the genetic defect to the offspring when needed (Practice Committee of American Society for Reproductive Medicine, 2013).

Hormonal stimulation protocol is determined by patient's age and ovarian reserve, but also by the available time before cancer treatment initiation. Ovarian hyperstimulation syndrome (OHSS) must be avoided, because it could significantly delay chemotherapeutic treatment and compromise prognosis. Therefore, the standard protocol for fertility preservation in patients with cancer includes stimulation with a Gonadotropin-releasing hormone (GnRH) antagonist protocol, and final maturation induction with a GnRH agonist, which is known to reduce the risk of OHSS compared to a human chorionic gonadotropin (hCG) trigger (Oktay et al., 2010). About 36 hours after trigger administration, oocytes are retrieved through a transvaginal ultrasound-guided pick-up.

Normally, ovarian stimulation should be started in the early follicular phase of the menstrual cycle, to stimulate a synchronous cohort of follicles and increase endometrial receptivity. However, to avoid delays in cancer treatment, random-start protocols have been introduced in the Oncofertility setting. In this way, stimulation can be started at any time during the menstrual cycle, reducing the time required to perform the procedure in patients who are candidates to chemotherapy (Sönmezer et al., 2011). Random start protocols have been shown to be efficient in providing a good number of mature oocytes

for cryopreservation with a significant reduction in the time needed for stimulation (Martínez et al., 2014).

In hormone-dependent tumors, such as breast cancer, an aromatase inhibitor (AI) can be added to the stimulation protocol to lower circulating estrogen levels and theoretically reduce the risk of enhanced proliferation of cancer cells (Rodgers et al., 2017). The role of estrogen in Estrogen Receptor (ER)-negative breast cancers is not yet clarified, however since there are some studies reporting a relation between estrogen-dependent factors and triple negative breast cancer, treatment with letrozole is currently recommended even in this subset of patients (Huang et al., 2015). The most commonly used AI is letrozole at the dose of 5 mg/day, administered daily during hormonal stimulation and discontinued on the day of the trigger. Some centers also used tamoxifen 60 mg daily concurrent administration with the same indication (Oktay et al., 2003). The pregnancy rates after embryo transfers using concurrent letrozole administration during COS were comparable to those obtained during in-vitro fertilization (IVF) cycles in a population without cancer (Oktay et al., 2015). There are some studies reporting a reduced oocyte yield in cycles that used the AI, due to the changes in the endocrine milieu at the follicular level (Revelli et al., 2013).

In a recent systematic review, including 15 studies, no detrimental effect on relapse-free survival was found in women with breast cancer undergoing COS with letrozole compared with breast cancer patients not undergoing fertility preservation (Rodgers et al., 2017). The largest long-term prospective study, including 337 women, found a hazard ratio for recurrence of 0.77. The subgroup analysis did not show any differences according to BRCA mutational status, ER status or neoadjuvant chemotherapy (J. Kim et al., 2016).

Interestingly, some studies have been published reporting a reduced number of retrieved oocytes after COS in patients with cancer even before receiving any chemotherapy, with respect to healthy patients of the same age (Friedler et al., 2012).

Quintero et al., (2010) showed that stimulation in oncological patients requires a higher total gonadotropin dose.

To explain these findings, some authors hypothesized that cancer itself might be detrimental on fertility, possibly due to an increased catabolic state, a form of hypothalamic dysfunction or a relative increase in stress hormone levels (Agarwal &

Said, 2004). In particular, patients with lymphoma showed a reduced ovarian reserve before the beginning of chemotherapy compared with healthy controls and patients with other cancers (Lekovich et al., 2016). No data is currently available for other cohorts of patients, such as patients with sarcomas.

Patients undergoing this fertility preserving strategy should be counselled that the pregnancy rates after oocyte cryopreservation are strictly dependent on the number of retrieved mature eggs. Poor egg retrieval makes a future pregnancy very unlikely.

3.3.2 Ovarian tissue cryopreservation

OTC is a fertility preservation technique suitable for patients who need to start chemotherapy urgently and cannot undergo hormonal stimulation. Additionally, it is the only available option in pre-pubertal patients (S. Kim et al., 2018).

The first human live birth from auto-transplantation of ovarian cortex has been reported in 2004 (Donnez et al., 2004), and since then the technique has progressively improved, reaching a 30-40% live-birth rate in the larger case series, both from spontaneous conception and assisted reproductive techniques (ART) (Poirot, Brugieres, et al., 2019), with more than 130 live births reported to date.

Until recently, this technique was still considered experimental by the major societies of reproductive medicine; however, common consensus is that it is now considered an acceptable procedure for fertility preservation (The ESHRE Guideline Group on Female Fertility Preservation et al., 2020).

Ovarian tissue can be retrieved by laparoscopy or mini-laparotomy. It is possible to cryopreserve the whole ovary or fragments of ovarian cortex. In pre-pubertal patients undergoing highly gonadotoxic schemes, usually the whole ovary is removed.

Ovarian tissue is transported from the operating theatre to the laboratory as quickly as possible, on ice. Then, ovarian cortex is isolated from the medulla and usually cut into fragments with a thickness of 1-2 mm (Poirot, Brugieres, et al., 2019). This is a necessary step to ensure adequate perfusion of cryoprotectants within the ovarian cortex.

There are three common techniques for cryopreservation of ovarian tissue: vitrification, slow-freezing and ultra-rapid freezing (Bahroudi et al., 2022). Slow-freezing was the first technique adopted for fertility preservation. With this technique, formation of ice crystals within the tissue could mechanically damage cells. The principal advantage of vitrification, instead, is that the cells solidify without formation of ice crystals (Shi et al., 2017).

Ovarian tissue can be transplanted back in a heterotopic or an orthotopic position. Heterotopic transplantation sites include the abdominal wall, rectus muscle, subperitoneal, forearm, o chest wall. This approach is mainly aimed at endocrine function restoration (for the treatment of menopausal symptoms or puberty induction), while orthotopic transplants to the ovary/pelvis are the most successful for reproductive purposes. Fragments can be placed on the ovary itself or in a peritoneal pouch and fixed with fibrin glue. A procedural variant is cryopreservation of the entire ovary and reimplant by a vascular anastomosis (Silvestris et al., 2020). One of the main issues with this technique is the follicular loss that can occur due to an ischemia-reperfusion damage and follicle activation after transplantation (Roness & Meirow, 2019).

The endocrine function is restored in 95% of cases (Gellert et al., 2018), and ovarian function is restored after 60-240 days from transplantation of the frozen-thawed tissue up to 7 years depending on patient age at the time of retrieval and amount of transplanted tissue (S. S. Kim, 2012). Transplantation can be also done in multiple surgeries to prolong the duration of endocrine production.

Unlike cryopreservation of oocytes, OTC can be theoretically performed in patients who already started gonadotoxic treatments, due to the lower risk of genetic abnormalities arising in the primordial follicle pool, on which this technique relies.

Data on this matter are scarce. Births after transplantation of chemotherapy-exposed tissue have been reported and there is a case series reporting good ovarian function recovery and reproductive outcomes following transplantation of tissue that had been harvested post-chemotherapy (Meirow et al., 2005)(Poirot, Fortin, et al., 2019).

A huge concern with OTC is the risk of contaminating malignant cells in the ovarian tissue specimen. The diagnoses that carry the higher risk in this sense are leukemia, Burkitt lymphoma, neuroblastoma and ovarian cancer (Silvestris et al., 2020).

The analysis of 5571 autopsies in young women with cancer under the age of 40 showed ovarian metastases in 22% of cases (Kyono et al., 2010). The authors concluded that reliable methods to detect minimal residual malignant disease in ovarian grafts are urgently needed.

So far, no relapses following ovarian tissue auto-transplantation have been reported. However, the theoretical risk of reseeding has been confirmed by animal xenografting studies using nude mice, showing that hematological malignancies can settle in the ovary. Therefore, even though in recent years ovarian transplantation has been successfully reported in acute leukemia patients (Shapira et al., 2018)(Sonmezer et al., 2020), the technique should be cautiously avoided in this group. Some authors have suggested that the technique can be performed after an initial round of chemotherapy to clear out the graft of malignant cells (Meirow et al., 2016). In the future, OTC may be suitable also to patients with ovarian metastases thanks to in vitro maturation (IVM) of oocytes and artificial ovary techniques (Hayashi et al., 2012). In vitro culture of isolated follicles obtained from cryopreserved ovarian cortex is not yet available to the stage of mature eggs, however experiments have been conducted using plasma clots in mice or a matrix of calcium alginate to create a suspension graft free of cancer cells (Dolmans et al., 2007)(Amorim et al., 2008).

3.3.3 Ovarian transposition

Primordial follicles appear to be very sensitive to radiation. Gonadal damage depends on the total dose, field of treatment, fractionation schedule, and patients' age. Amenorrhea occurs with a radiation dose ≥ 6 Gy in adult women, ≥ 10 Gy in post-pubertal girls, and ≥ 15 Gy in prepubertal girls (Lee et al., 2021).

Ovarian transposition is a procedure in which the ovaries are relocated outside the radiation field, in women of fertile age who are candidates to pelvic radiotherapy. It is recommended in women younger than 40 years, who are not at high risk of ovarian metastases and that are not candidates to gonadotoxic chemotherapy (Moawad et al., 2017).

The principal indications in adults include cervical and rectal cancers, whereas in children ovarian transposition is performed in case of rhabdomyosarcoma of the bladder, vagina or uterus, and soft tissue or pelvic bone sarcomas (Irtan et al., 2013). The procedure can be performed by laparoscopy, and concomitant ovarian tissue cryopreservation may be considered. In the most common variant, when a midline radiation field is planned, ovaries are mobilized by transecting the utero-ovarian ligament and moved cranially and laterally to the anterolateral abdominal wall. Instead, if the tumor is lateral, the homolateral ovary is moved to the other side of the pelvis. Usually, they are marked by metallic clips intraoperatively so that they can be identified by radiologic examinations (Irtan et al., 2013).

A small number of pregnancies after ovarian transposition have been described, both spontaneous and from ART (Terenziani et al., 2009). However, pelvic irradiation may also be detrimental to the uterus so that, depending on dose, a successful pregnancy might be unlikely in many of these patients, even when ovarian function is preserved. Pregnancy would then only be possible resorting to a gestational surrogate (Giacalone et al., 2001).

3.4 Fertoprotective agents

3.4.1 Ovarian function suppression

The use of GnRH agonists (GnRH-a) during chemotherapy is still a controversial method of fertility preservation. The possibility of a non-invasive and non-expensive pharmacological treatment administered during chemotherapy to reduce ovarian damage is indeed very appealing.

The principal side effects of GnRH-a treatment include symptoms related to hypoestrogenism, such as hot flashes, vaginal dryness, mood swings, and insomnia. Treatment-associated amenorrhea may be beneficial in case of abnormal uterine bleeding during oncological treatments, especially in hematological malignancies.

Historically, the rationale of this strategy relied at first upon the observation that a decreased gonadal activity during gonadotoxic treatment can be associated with a less

severe degree of ovarian damage, i.e. what is observed in pediatric populations (Rivkees & Crawford, 1988). Of course, the lower rates of ovarian failures in this population are partially explained by the larger ovarian reserve in childhood. However, the hypogonadotropic prepubertal milieu might be favorable in this respect according to some authors (Blumenfeld, 2019), and many studies tried to explore this subject.

GnRH-a binds GnRH-a receptors on the anterior pituitary. Initially, GnRH-a activates receptors, stimulating secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Prolonged activation of the receptors results in desensitization and downregulation of gonadotropin secretion, which should result in reduced follicular activity at the ovarian level. This could prevent the increased rate of folliculogenesis that is observed after a gonadotoxic insult (Blumenfeld, 2019). Blumenfeld and von Wolff proposed the theory that GnRH-a could interfere with the follicular burnout phenomenon by blocking the vicious circle of activation of many follicles simultaneously due to the local decrease in AMH and subsequent increase in follicular recruitment through FSH secretion (Blumenfeld & von Wolff, 2008).

Detractors of GnRH-a concurrent administration as a fertility preservation strategy during chemotherapy state that suppressing oocyte maturation by GnRH-a does not prevent damage to primordial follicles, which ultimately constitute the ovarian reserve, because their activation is not dependent on gonadotropin stimulation (Dolmans et al., 2020).

The gonad protective effect could also be due to an activation of an anti-apoptotic pathway in granulosa cells (Scaruffi et al., 2019). This could happen through up-regulation of ovarian protecting molecules like sphingosine-1-phosphate. Several studies showed a protective effect of this molecule on follicles by a reduction in doxorubicin-induced and radiation-induced apoptosis (Morita et al., 2000)(Zelinski et al., 2011). However, there is currently no evidence that GnRH-a increases the secretion of such a molecule, and this is currently just speculation.

A possible direct ovarian effect has also been postulated. GnRH receptors are expressed by the gonads, as seen in rodent studies. Imai et al. showed that Buserelin decreases the toxicity of doxorubicin in vitro through a local mechanism (Imai et al., 2007). The role of ovarian GnRH receptors is not fully understood: in some conditions, they may have an inhibitory role on immature follicles inhibiting steroidogenesis,

whereas on mature follicles, instead, they seem to promote maturation (Whitelaw et al., 1995).

Another mechanism hypothesized in the literature, that could have a gonad protective effect, is a reduction in the ovarian blood flow which is regulated by estrogen levels (Hickman et al., 2016). This could theoretically diminish ovarian exposure to the gonadotoxic drug in a pituitary-dependent manner. In rats, treatment with GnRH-a inhibited the estrogen-induced increase in ovarian perfusion (Kitajima et al., 2006). However, some argue that this effect might be detrimental and potentially increase ovarian damage and fibrosis instead of being beneficial (Hasky et al., 2015).

Some authors have postulated that GnRH-a induced hypogonadotropic milieu might be protective of undifferentiated germinative stem cells, whose existence is still matter of debate. This might explain the reprisal of ovarian activity seen in some women that showed menopausal FSH levels and undetectable AMH levels after a year or more by the end of chemotherapy (Blumenfeld, 2019).

Figure 3 summarizes the hypothesized indirect and direct effects of GnRH-a as a gonad protectant during chemotherapy.

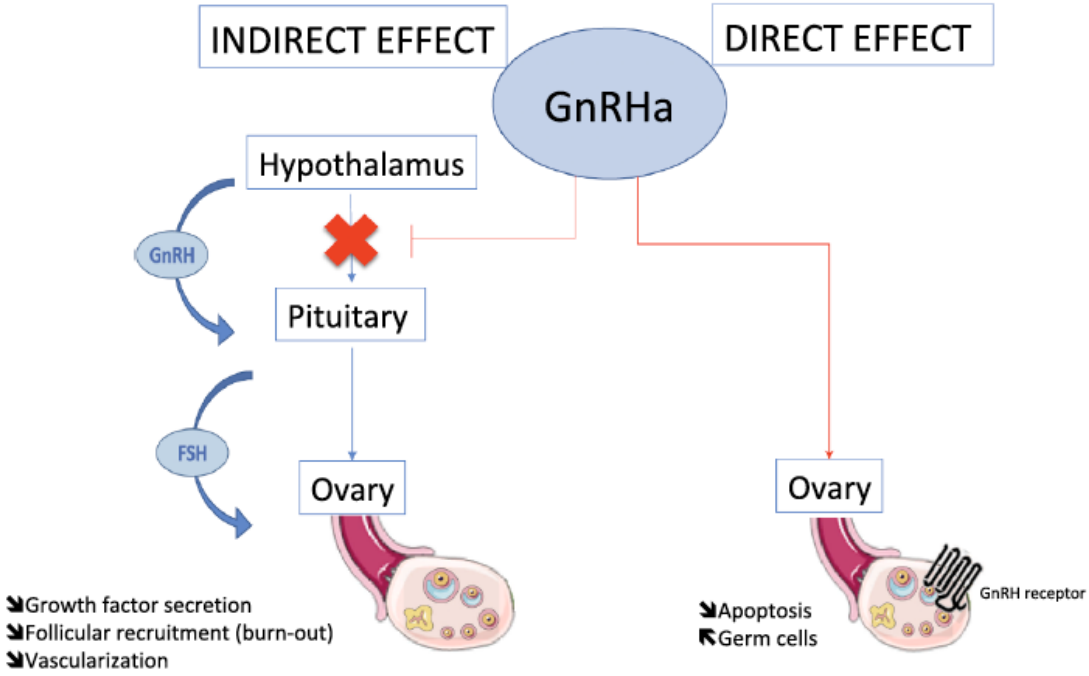

Figure 3. *Indirect and direct mechanisms for the gonadal protection of GnRH agonists during chemotherapy (from (Lambertini et al., 2019 according to "fair use principle", for educational purposes only)*

Despite favorable pre-clinical evidence (Glode, 1981), GnRH-a co-treatment is not effective for the protection of reproductive function in males (Johnson et al., 1985).

Many clinical trials have been conducted on the topic of GnRH-a as gonad protectants during chemotherapy. To date, we can count 14 randomized controlled trials, 25 non-randomized trials and 20 meta-analyses (Dolmans et al., 2020).

The majority of clinical data on GnRH-a use during chemotherapy can be found in the breast cancer patients' population. Some trials focused on hematological malignancies, while very limited data are currently available for other solid tumors.

GnRH is now part of the oncological treatment of high-risk estrogen receptor-positive cancers (Lambertini, Boni, et al., 2015)(Francis et al., 2018). For this reason, menstrual function resumption is not easy to study in the breast cancer population due to the large use of hormone therapy maintenance after chemotherapy.

In the Prevention of Early Menopause Study (POEMS) the overall survival of patients treated with GnRH-a plus chemotherapy was significantly higher than that of patients treated with chemotherapy alone (Moore et al., 2015). This trial only enrolled women with hormone receptor negative breast cancer, so the result was unexpected. Women

treated with GnRH-a had a reduction in ovarian failure rate at 2 years (OR 0.30, 95% CI 0.09-0.97; P=.04) and a higher pregnancy rate than those in the chemotherapy alone group (OR 2.45; 95% CI 1.09-5.51; P=.03). There may be several biases to this result, because many confounding factors can impact pregnancy rates, especially in oncological patients.

In the PROMISE trial, 281 patients with breast cancer were randomized to receive chemotherapy with or without monthly injections of triptorelin starting 1 week before the beginning of gonadotoxic treatments: the reported rate of early menopause in treated women was 8.9% vs 25.9% without treatment (Del Mastro et al., 2011). The principal difference with the other trial is that the majority of patients enrolled in the PROMISE trial had hormone receptor-positive disease. In this trial, the 12-year cumulative incidence of pregnancy was higher in the GnRH-a group, but did not reach statistical significance. The number of pregnancies among women enrolled in the PROMISE trial was lower than those enrolled in the POEMS study, probably because of the need of adjuvant endocrine therapy in the hormone receptor positive subgroup for 5-10 years after chemotherapy (Lambertini et al., 2022). This study is the one with the longest follow-up, showing a 5-year cumulative resumption of menses of 72.6% in the GnRH group compared to 64% among controls, with a comparable disease-free survival (DFS).

A Chinese randomized trial reported similar results to those of the PROMISE trial (Song et al., 2013).

In the OPTION trial, the primary outcome was amenorrhea 12-24 months after randomization with elevated FSH levels. The GnRH-a goserelin showed a benefit in preventing amenorrhea after chemotherapy, even though a marked reduction in AMH serum levels in both cohorts was noted, raising doubts about efficacy in prevention of chemotherapy-related infertility (Leonard et al., 2017).

To note, there were also some trials that had to stop recruiting prematurely for futility as the ZORO trial, that showed no evidence of benefit for GnRHa administration and high rates of menses recovery after chemotherapy for both arms (Gerber et al., 2011). This could be also due to the younger median age of patients enrolled (around 35 years old). For studies enrolling patients receiving low gonadotoxic protocols, POF rates are so low that a huge number of patients would be required to detect a significant effect on ovarian protection. Therefore, studies in these cohorts are not easy to conduct (Blumenfeld et al., 2014).

Conversely, an Egyptian trial enrolling hormone-receptor negative breast cancer patients concluded for no benefit for GnRH-a administration with respect to menstrual function resumption (E. A. Elgindy et al., 2013).

Figure 4 summarizes the randomized controlled trials (RCT) conducted on the gonad-protective role of GnRH-a in breast cancer.

AUTHORS	POI DEFINITION (TIMING)	NUMBER OF PATIENTS	MAIN RESULTS (GNRHA VS CONTROL)	PROTECTION
Li et al[32]	Amenorrhea (12 months)	63	• POI rate: 32.1% vs 53.1% ($P = .027$)	YES
Badawy et al[33]	Amenorrhea and no resumption of ovulation (8 months)	78	• POI rate: 11.4% vs 66.6% ($P < .001$)	YES
Sverrisdottir et al[34]	Amenorrhea (up to 36 months)	94	• POI rate: 64% (93%) vs 90% (87%) ($P = .006$)	YES
Gerber et al[35]	Amenorrhea (6 months)	60	• POI rate: 30% vs 43.3% ($P = .142$) • Pregnancies: 1 vs 1	NO
Sun et al[36]	Amenorrhea (12 months)	21	• POI rate: 27.3% vs 50.0% ($P = .039$)	YES
Del Mastro et al[37] and Lambertini et al[44]	Amenorrhea and post-menopausal FSH and E2 levels (12 months)	281	• POI rate: 8.9% vs 25.9% ($P < .001$) • Pregnancies: 8 vs 3 ($P = .20$)	YES
Munster et al[38]	Amenorrhea (24 months)	49	• POI rate: 15% vs 14% ($P = .32$) • Pregnancies: 0 vs 2	NO
Elgindy et al[39]	Amenorrhea (12 months)	100	• POI rate: 20%/16% vs 20%/20% ($P = 1.00/P = .71$) • Pregnancies: 2 vs 1	NO
Song et al[40]	Amenorrhea and post-menopausal FSH and E2 levels (12 months)	183	• POI rate: 16.9% vs 28.7% ($P < .01$)	YES
Jiang et al[41]	Amenorrhea (NR)	21	• POI rate: 10.0% vs 45.5% ($P = .05$)	YES
Karimi-Zarchi et al[42]	Amenorrhea (6 months)	42	• POI rate: 9.5% vs 66.7% ($P < .001$)	YES
Moore et al[43] and Moore et al[47]	Amenorrhea and post-menopausal FSH levels (24 months)	218	• POI rate: 8% vs 22% ($P = .04$) • Pregnancies: 23 vs 13 ($P = .04$)	YES
Leonard et al[45]	Amenorrhea and post-menopausal FSH levels (between 12 and 24 months)	221	• POI rate: 18.5% vs 34.8% ($P = .048$) • Pregnancies: 7 vs 5	YES
Zhang et al[46]	Amenorrhea and post-menopausal FSH and E2 levels (36-72 months)	216	• POI rate: 23.1% vs 22.8% ($P = .969$)	NO

Abbreviations: E2, estradiol; FSH, follicle-stimulating hormone; GnRHa, gonadotropin-releasing hormone agonist; NR, not reported; POI, premature ovarian insufficiency.

Figure 4. *Summary of RCTs on the gonad protective role of GnRH-a in breast cancer patients receiving chemotherapy (from Lambertini, Richard, et al., 2019, according to "fair use principle", for educational purposes only).*

Even though mixed conclusions can be inferred from trial results, meta-analyses of ovarian protection by GnRH-a in the setting of breast cancer favored the use of these compounds during chemotherapy with the purpose of preventing post-treatment amenorrhea and, to some extent, POF, although with high heterogeneity (Munhoz et al., 2016)(Lambertini, Ceppi, et al., 2015)(Silva et al., 2016) (Bai et al., 2017). The majority of these meta-analyses include overlapping studies and some data deriving from non-randomized trials, so that the benefit may be overestimated (Blumenfeld et al., 2014). Few studies report the pregnancy intent, which could bias pregnancy rates especially in non-blinded, non-placebo controlled trials on fertility preservation (Turan et al., 2019). In fact, post-treatment pregnancies were included as a pre-planned study endpoint only in the POEMS trial (Lambertini, Horicks, et al., 2019).

There are still no data on the efficacy of this strategy in the subset of breast cancer patients carrying a germline BRCA mutation.

A meta-analysis of randomized trials enrolling women with breast cancer not treated with tamoxifen did not show any benefit of GnRH-a use in preventing POF by analyzing differences in menses resumption after 1 year (Vitek et al., 2014).

The conflicting results can be partially explained by the different definition of outcomes among trials and meta-analyses, especially the timings of amenorrhea definitions. Also, another diverting element is the upper age limit for trial inclusion. Several studies enrolled women over 40 years, whose ovarian reserve is so low that a difference in fertility preservation is probably not detectable, at least with the numbers considered in most trials.

Mostly negative results have been published in the setting of hematological malignancies. In a trial including 129 patients with lymphoma (Hodgkin and non-Hodgkin), no added benefit was reported for GnRH-a administration in the prevention of chemotherapy-induced POF (Demeestere et al., 2016). However, in these patients GnRH agonist could be administered only 2 days before chemotherapy initiation, due to the necessity to start treatment urgently, which is typical of this diagnosis. Another possible explanation for these different results in the lymphoma cohort is the wide variety of gonadotoxicity of the chemotherapy regimens used in this setting (either very low i.e.

ABVD vs very high i.e. BEACOPP) compared to the regimens used for breast cancer, generally considered of intermediate toxicity.

Only one small, randomized trial has been conducted in the setting of ovarian cancer, including a total of 30 patients. Six months by the end of treatment, all women in the GnRH-a arm had regular menstrual periods, while 33% of the women treated by chemotherapy only had amenorrhea (Lambertini, Horicks, et al., 2019).

Meta-analyses comprehensive of different cancer types in addition to breast cancer were not conclusive for a benefit on ovarian function and subsequent pregnancies (E. Elgindy et al., 2015).

Figure 5 summarizes the RCTs conducted on the gonad-protective role of GnRH-a in hematological malignancies.

Table 3. Summary of the RCT using GnRH-a in parallel to chemotherapy for fertility preservation in hematologic diseases

Study	# of patients	Median age	Treatment	Treatment start	End point/ objective	Multivariable analysis	End point difference (%)	Time	Cumul. Cycloph.
Behringer et al. [35]	19	26 (18–40)	Escalated BEACOPP	7 days	AMH		POF 30/55.6	18 months	10.0 g/m^2
Behringer et al. [36]	331	18–40	Esc BEACOPP + ABVD	7 days	Pregnancies	Yes	Pregnancies 36/16	42 months	
Demeestere et al. [37] 2013	84	26 (18–45)	Polychemotherapy	10 days	AMH		POF 20/19 AMH 1.4/0.5	1 year	5.3 g/m^2

POF, premature ovarian failure; AMH, anti-Mullerian hormone. 'Time' refers to the time after chemotherapy at evaluation of POF and menstrual cyclicity; Ctrl, control. In the 'end point difference' column, the report refers to the figures in the GnRH-a/control groups, respectively.

Figure 5. *Summary of RCTs on the gonad protective role of GnRH-a in hematological malignancies (from Blumenfeld et al., 2014, according to "fair use principle", for educational purposes only).*

All the studies that have been conducted on this topic are limited by heterogeneity of populations, treatments and outcome definitions. Particularly, diverse surrogate endpoints of ovarian function have been used, including recovery of menses, hormone serum levels and ovarian reserve markers. Most of the trials on this subject are insufficiently powered due to small populations, high drop-out rate, and short follow-ups. Menses resumption can occur up to 2 years after chemotherapy, so that early assessments can underestimate the effect of GnRH (Dolmans et al., 2020). Only a minority of trials compared pregnancy rates between patients using gonadal protection and patients not using it, so that the statistical power is limited. Another important aspect is that very long follow ups would be required to verify whether patients receiving GnRH with chemotherapy and resuming menses experience early menopause after a few years or the benefit is maintained over the course of time. This is true especially for the cohort of

lymphoma patients, that are generally very young at the time of diagnosis and therefore tend to resume menses after treatment irrespectively of GnRH-a administration, but may experience later in life a premature onset of menopause as a consequence of the gonadotoxic insult.

The Cochrane meta-analysis concluded for a benefit in protecting menstrual function and ovulation after chemotherapy (Chen et al., 2011).

Despite conflicting evidence, GnRH-a concurrent administration has been integrated in international guidelines in addition to more established fertility preservation strategies (Oktay et al., 2018), at least in the breast cancer setting where the evidence in favor is more robust.

Pre-clinical studies on GnRH-a use as a gonad protectant have not been conclusive.

Initial studies seemed to favor its use concomitant to chemotherapy administration, but subsequent experiments did not confirm previous findings, so that the mechanisms of action remain unclear.

The majority of pre-clinical studies have been conducted using mice and rats.

Regarding the capacity of GnRH agonists to inhibit HPG axis in the murine model, evidence is conflicting. Some studies reported no changes in FSH levels in rats after GnRH-a administration, while others showed reduced estradiol serum levels (Horicks et al., 2015).

Horicks et al. (2018) showed that triptoreline failed to inhibit folliculogenesis in mice and did not reduce FSH levels; a protective effect on follicular demise after administration of cyclophosphamide was not confirmed. Additionally, cyclophosphamide-induced ovarian damage was not inhibited in a FSH-deficient mouse model, questioning the role of HPG inhibition in the gonadoprotective mechanism of GnRH-a (Horicks et al., 2015). Detti et al. showed no inhibition of cyclophosphamide induced follicular depletion after administration of goserelin (Detti et al., 2014).

In an experiment using doxorubicin, GnRH-a failed to protect total follicles and AMH levels. The authors hypothesized that the reduced vascularity induced by GnRH resulted in a reduced delivery of Vascular Endothelial Growth Factor (VEGF) with decreased neovascularization after chemotherapeutic injury (Hasky et al., 2015).

Yuce et al. observed a protective effect of GnRH-a on follicular loss after exposure to high doses of cyclophosphamide (100 mg/kg) but not at lower doses. Some level of follicular loss was observed in any case irrespective of GnRH-a administration (Yüce et al., 2004).

Other studies showed a dose-dependent protection of triptorelin on murine follicular counts after exposure to cyclophosphamide and busulfan (Kishk & Mohammed Ali, 2013) (Tan et al., 2010), while others showed a protective effect of goserelin against cisplatin (Lin et al., 2012) and leuprolide against docetaxel (Park et al., 2017).

An inhibitory effect on recruitment of quiescent follicles was also observed in studies using a rat model of gonadotoxicity (K. M. Ataya et al., 1985). Conflicting results derived from later studies demonstrating that anovulation did not prevent cyclophosphamide-mediated follicle loss (Letterie, 2004). Other studies suggested a protective effect against paclitaxel toxicity but not against cisplatin (Ozcelik et al., 2010).

Few data are available in primates and human cell models.

A prospective randomized study with histological assessment has been conducted in Rhesus monkeys using cyclophosphamide: it showed an increased primordial follicle demise in monkeys treated with cyclophosphamide without concurrent GnRH-a administration (K. Ataya et al., 1995).

Imai et al. showed preserved estradiol levels in human granulosa cells exposed in vitro to doxorubicin after concurrent GnRHa administration (Imai et al., 2007)). A more recent study using human granulosa cells cultured in vitro did not show any protection from exposure to cyclophosphamide, paclitaxel, 5-fluorouracil and TAC combination regimen (docetaxel, adriamycin and cyclophosphamide) in terms of follicular counts, hormone levels, anti-apoptotic genes and vascularity (Bildik et al., 2015).

All these discrepancies among pre-clinical trials can be explained by the different experimental models, chemotherapy regimens and doses, and type, dose and mode of administration of the GnRH-a used (Lambertini, Horicks, et al., 2019).

One of the limits of the murine model in this field is that estrous cycle differs from the human cycle and mixed evidence can be inferred on the GnRH-a mediated suppression of the HPG axis (Horicks et al., 2015). The direct action of GnRH-a in murine and human ovaries has been poorly studied and there could be differences depending on the species considered (Lambertini, Horicks, et al., 2019).

Figure 6 summarizes pre-clinical evidence of GnRH-a gonad-protection in murine models.

Authors	Type of gonadotoxic treatment	Main results	Overall results
Yuce et al., 2004	Cyclophosphamide	* Small protection of primordial follicles	Protection (only against high dose of cyclophosphamide)
Danforth et al., 2005; Kishk et al., 2013; Hasky et al., 2015; Kanter et al., 2016	Cyclophosphamide	* Dose-dependant protection of the ovarian reserve * Slight protection of growing follicles * Preservation of AMH levels * Preservation of fertilization rate, early embryo development and improvement of oocyte quality	Protection
Tan et al., 2010	Busulfan	* Protection of primordial and primary follicles	Protection
Lin et al., 2012; Zhang et al., 2013	Cisplatin	* Protection of quiescent and growing follicles * Preservation of AMH levels * No difference in proliferation and apoptosis in the ovaries	Protection
Detti et al., 2014; Horicks et al., 2015; Horicks et al., 2018	Cyclophosphamide	* No protection of quiescent and growing follicles * No protection of FSH and AMH levels * FSH deficiency does not protect ovarian reserve * *In vitro* exposure to GnRHa does not preserve follicular survival * No difference in proliferation and apoptosis in the ovaries	No protection
Hasky et al., 2015	Doxorubicin	* Compromise vascular recovery * No preservation of AMH levels	No protection
Park et al., 2017	Docetaxel	* Protection of total follicles * Preservation of proliferation within follicles	Protection

Figure 6. *Preclinical studies of GnRH-a gonad protection in murine models (from Lambertini, Horicks, et al., 2019 according to "fair use principle", for educational purposes only).*

4. Aim of the work

This is a project investigating the following fertility preservation strategies in women with cancer: GnRH-a administration, oocyte cryopreservation and OTC.

The project's specific aims were:

- to investigate the potential protective effects of concurrent GnRH-a administration in a Contrast-Enhanced Ultrasound (CEUS)-based murine model of gonadotoxicity, in combination with two different chemotherapeutic drugs (cyclophosphamide and doxorubicin);
- to evaluate the opportunity to perform OTC in post-pubertal patients who already received chemotherapy;
- to identify risk factors for suboptimal response to COS in breast cancer patients;
- to evaluate outcomes of COS in the population of young patients diagnosed with a sarcoma;
- to identify factors associated with a higher risk of POF in oncological patients;
- to develop an ultrasound-based protocol to characterize chemotherapy-related uterine damage in young women diagnosed with cancer.

5. Results

5.1 Murine studies

5.1.1 Characterizing GnRH agonists' action in a murine model of gonadotoxicity: ultrasound and hormonal findings

In the first set of experiments, 14-weeks old BALB/c mice (Charles River) were treated with a subcutaneous injection of a GnRH-a, 100 ul of Decapeptyl SR (Ipsen, Milan, Italy) equivalent to 4.45 mg/kg (n=7, experimental group) or of 100 ul of saline (0.9% NaCl) sterile solution (n=7, control group).

For the whole duration of the study, GnRH-a treated mice and controls were in a healthy status and did not lose weight.

US assessments

At day 14 after GnRH-a administration, ovarian volume was higher in treated mice compared with controls (4.494 ± 0.772 mm^3 in GnRH-treated vs 3.139 ± 1.489 mm^3 in controls; P = 0.0538). The diameter of the dominant follicle increased significantly in treated mice compared with controls (0.696 ± 0.132 mm in GnRH-treated vs 0.267 ± 0.041mm in controls; P **** = 0.0001) (**Table 1**).

	DAY 0			**DAY 14**		
	GnRH-a treated (n=7)	Control (n=7)	p-value	GnRH-a treated (n=7)	Control (n=7)	p-value
Ovarian Volume (mm^3)	2.755 ± 0.585	2.807 ± 0.896	0.900	4.494 ± 0.772	3.139 ± 1.489	0.0538
Dominant Follicle Diameter (mm)	0.288 ± 0.107	0.235 ± 0.056	0.264	0.696 ± 0.132	0.267 ± 0.041	< 0.0001 (****)

Table 1. *Variation of ultrasound parameters in GnRH-a treated mice versus controls.*

Data are presented as mean ± standard deviation.

**, P < 0.05; **, P < 0.005, ***, P < 0.0005 and ****, P < 0.0001 by t test.*

CEUS (Contrast-Enhanced Ultrasound) assessments

CEUS analysis was successfully performed (quality of fit, QOF > 70%) in 5/7 cases for the GnRH-a group and 6/7 cases for the control group.

At day 14, no significant differences could be identified in perfusion parameters between treated mice and controls (**Figure 7)**; however, the Peak Enhancement (PE), Wash-in rate (WiR), Wash-in Perfusion index (WiPi) and Area under the curve (AUC) variance values of the 2 groups resulted significantly different (**Table 2**).

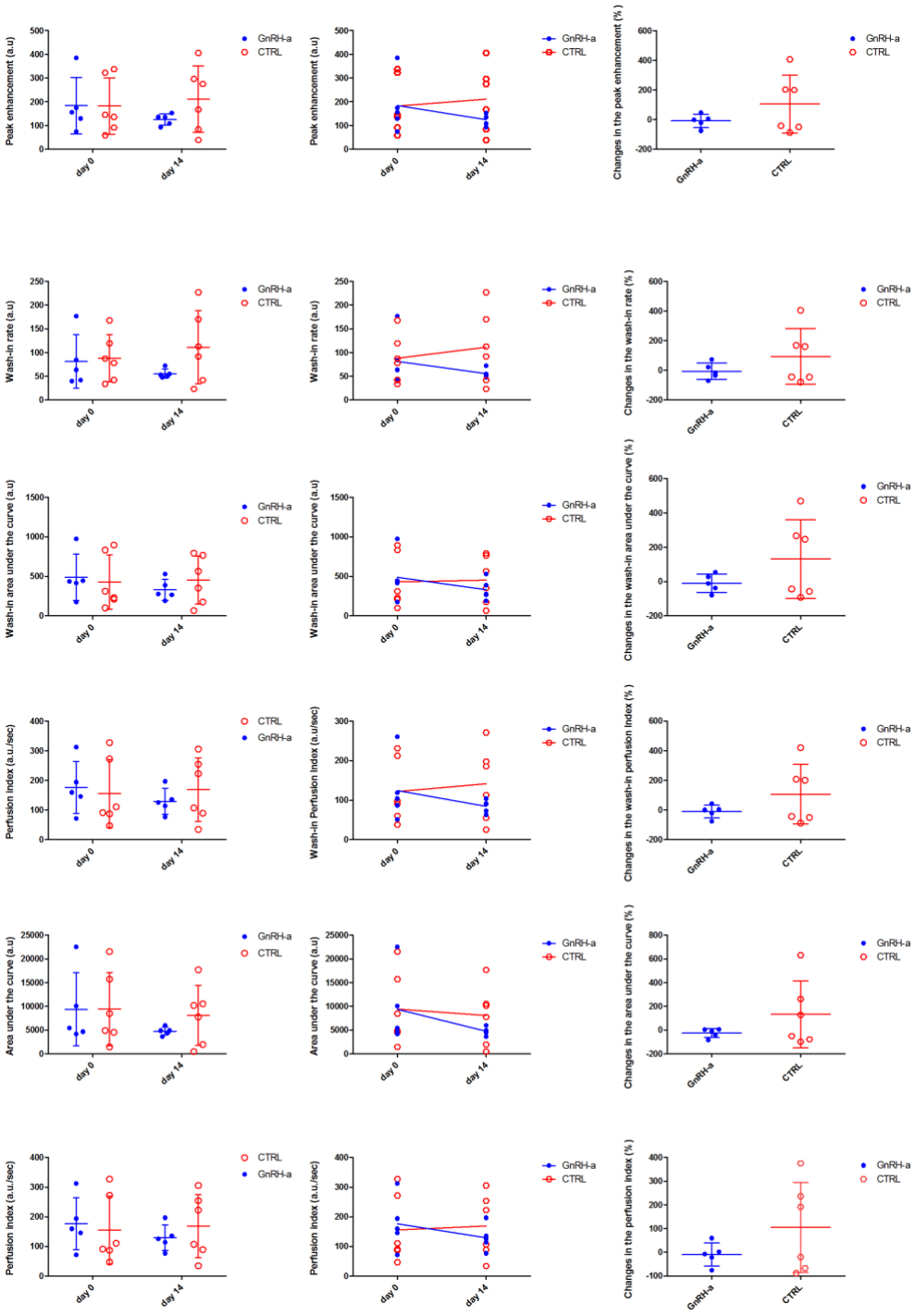

Figure 7. *Quantitative analysis of Contrast Enhanced Ultrasound (CEUS) imaging of the right ovary, performed in GnRH-a treated (n=5, blue plots) and control mice (n=6, clear plots) at day 0 and day 14 after treatment. Perfusion parameters (peak enhancement, PE; wash-in rates, WiR; wash-in area under the curve, WiAUC; wash-in perfusion index, WiPI; area under the curve, AUC, perfusion index, PI) were calculated and plotted on the scatter graphs (graphs on the left) (Bars: mean ± SD). The trends from day 0 to day 14 is presented in graphs in the middle (lines: trend of the average; blue line: GnRH-a treated group; red line: control group). The analysis* of *the average percentage change in perfusion parameters pre-to-post treatment is presented in the graphs on the right.*

	DAY 0			*Table 2*	***DAY 14***			
	GnRH-a Treated mice (n=5)	***Control Untreated mice (n=6)***	*t*-test p-value [a]	*F*-test p-value [b]	***GnRH-a Treated mice (n=5)***	***Control Untreated mice (n=6)***	*t*-test p-value [a]	*F*-test p-value [b]
PE [A.U.]	183.8 ± 118.8	181.7 ± 119.0	**0.9770**	**1.000**	*124.9* ± 23.57	211.0 ± 139.6	**0.2094**	**0.0043****
WIR [A.U.]	81.12 ± 56.40	88.07 ± 50.08	**0.8334**	**0.7843**	55.58 ± 9.458	111.1 ± 77.24	**0.1479**	**0.0012****
TTP [S]	4.710 ± 0.9197	4.043 ± 0.8057	**0.2316**	**0.7635**	4.572 ± 0.9460	3.823 ± 0.5771	**0.1398**	**0.3074**
RT [S]	3.976 ± 0.6786	3.258 ± 0.6396	**0.1048**	**0.8775**	3.810 ± 0.8607	3.125 ± 0.5261	**0.1380**	**0.3093**
WIAUC [A.U.]	487.6 ± 293.5	428.7 ± 343.4	**0.7695**	**0.7837**	328.8 ± 131.4	451.6 ± 303.9	**0.4251**	**0.1298**
WIPI [A.U. S $^{-1}$]	124.2 ± 80.35	121.7 ± 80.58	**0.9604**	**1.000**	84.06 ± 16.42	141.3 ± 93.44	**0.2125**	**0.0050****
MTT [S]	52.14 ± 22.97	56.68 ± 18.48	**0.7244**	**0.6375**	39.01 ± 8.921	43.03 ± 24.45	**0.7374**	**0.0734**
AUC [A.U.]	9384 ± 7725	9428 ± 7704	**0.9927**	**0.9670**	4770 ± 850.1	8096 ± 6302	**0.2755**	**0.0018****
PI [A.U. S $^{-1}$]	176.7 ± 87.94	155.8 ± 114.7	**0.7466**	**0.6272**	129.4 ± 43.56	168.7 ± 107.0	**0.4646**	**0.1062**

Table 2. *CEUS analysis: Variation of perfusion parameters in GnRH-a treated mice versus controls.*

Data are presented as mean ± standard deviation.

a.u.: arbitrary units

[a] *Unpaired t-test p value*

[b] *F-test to compare variances p-value*

**, P < 0.05; **, P < 0.005 by t test and F test*

CEUS analysis with Advanced Contrast Quantification Software was successfully performed in 20/20 cases with 100% technical success at day 0 and day 14.

In GnRH-a treated mice, perfusion parameters became more homogeneous compared to the control group for PE, WiR, WiPi and AUC, from day 0 to day 14 (**Figure 7**; graphs at the right), with a trend towards lower values of perfusion (**Figure 7**; graphs in the middle). The analysis of the percentage change in perfusion parameters, pre-to-post treatment, also showed a statistically significant difference in variance between treated and untreated mice, for PE, WiR, WiAUC, WiPi, AUC and Perfusion Index (PI) (**Table 3**). In GnRH-a treated mice the percentage changes in PE, WiR,WiAUC, WiPi, AUC and PI, from day 0 to day 14, were more homogeneous compared to the control group, with lower perfusion values after 14 days (**Figure 7**; graphs at the left).

FROM DAY 0 TO DAY 14

	GnRH-a Treated mice(n=5)	***Control Untreated mice (n=6)***	*t*-test p-value [a]	*F*-test p-value [b b]
PE [%]	-9.842 ± 44.72	104.5 ± 196.2	0.2376	0.0134*
WIR [%]	-6.086 ± 54.96	93.25 ± 187.5	0.2856	0.0339*
TTP [%]	0.2660 ± 27.35	-2.986 ± 20.26	0.8408	0.5240
RT [%]	-1.576 ± 27.55	-1.262 ± 21.73	0.9835	0.6090
WIAUC [%]	-9.642 ± 53.11	131.2 ± 229.0	0.2149	0.0143*
WIPI [%]	-10.62 ± 43.46	107.2 ± 201.0	0.2342	0.0110*
MTT [%]	-15.90 ± 30.79	-9.678 ± 60.80	0.8410	0.2118
AUC [%]	-24.71 ± 38.04	132.5 ± 281.7	0.2506	0.0018**
PI [%]	-8.816 ± 48.32	104.8 ± 189.7	0.2281	0.0203*

Table 3. *Average percentage change in perfusion parameters pre-to post treatment in GnRH-a treated mice versus controls.*

Data are presented as mean ± standard deviation.

a.u.: arbitrary units

[a] Unpaired t-test P-value

[b] F-test to compare variances P-value

**, P < 0.05; **, P < 0.005 by t test and F test*

CEUS analysis with Advanced Contrast Quantification Software was successfully performed in 20/20 cases with 100% technical success at day 0 and day 14.

Serological assessments

In Decapeptyl-treated mice, FSH levels at day 7 after the injection were not significantly different compared to those of control mice (53.56 ± 19.72 mUI/ml vs 38.23 ± 24.85 mUI/ml; p=0.266). However, in treated mice, a reduction of FSH levels at day 12 after GnRH-a injection compared to baseline levels was observed (16.31 ± 4.33 mUI/ml vs 22.94 ± 7.25 mUI/ml, p=0.04*). At day 20, a trend for a reduction of FSH levels could still be observed (14.21 ± 1.79 mUI/ml vs 22.94 ± 7.25 mUI/ml, p=0.06).

Immunofluorescence and immunohistochemical assessments

We hypothesized that the stimulatory effects on folliculogenesis could be determined by a local effect of GnRH. To test this hypothesis, two commercially available antibodies against GnRHR were tested on 10 samples of murine ovarian tissue. A diffuse GnRHR immunoreactivity was identified in ovarian tissue from both Decapeptyl-treated mice (n=5) and controls (n=5) (**Figure 8**).

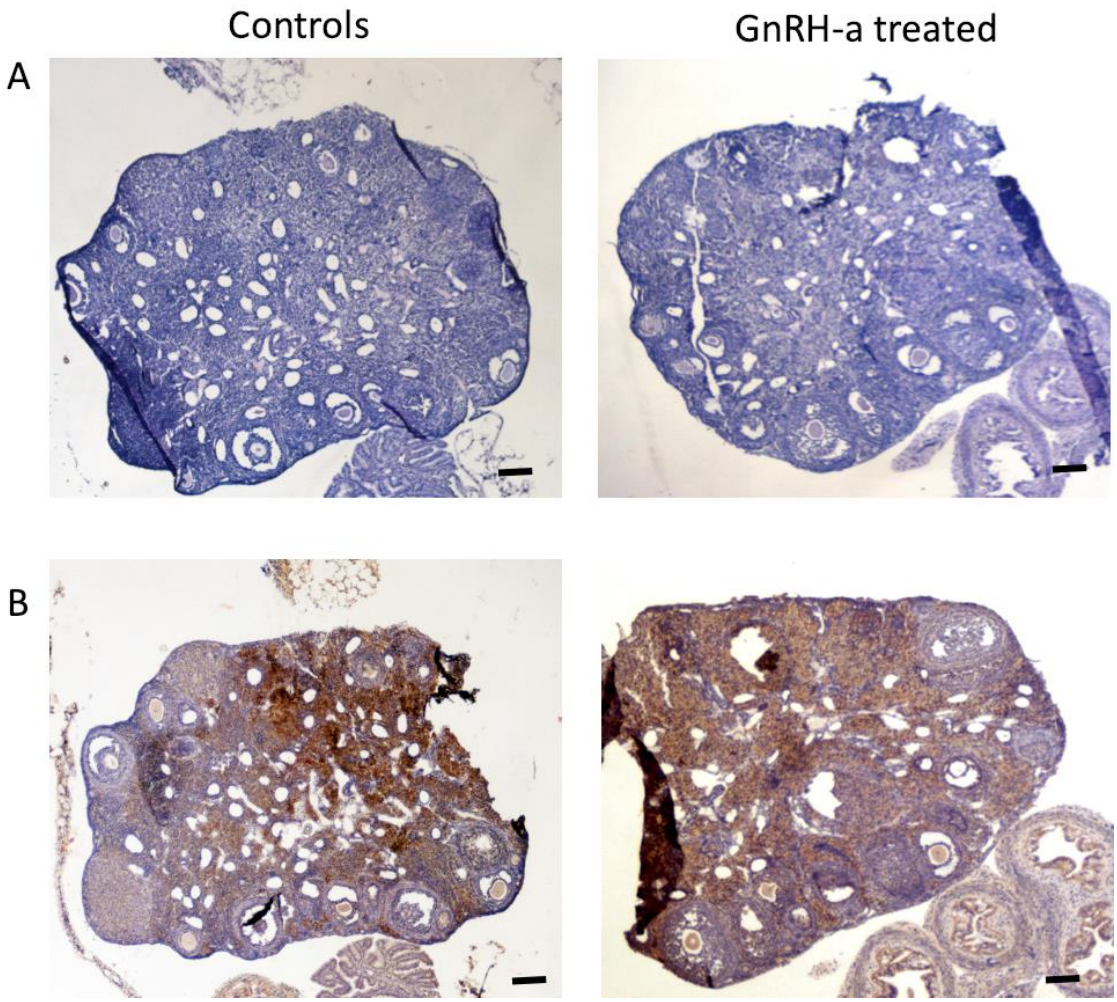

Figure 8. *Diffuse GnRHR immunoreactivity in ovarian tissue from both Decapeptyl-treated mice and controls (4x magnification; scale bar 100µm).*

A *H&E staining in control (left) and Decapeptyl-treated ovarian tissue (right)*

B *GnRHR staining in control (left) and Decapeptyl-treated ovarian tissue (right)*

Finally, ovarian tissue samples from Decapeptyl-treated mice (n=5) and controls (n=5) were stained with antibodies against blood-derived human endothelial cell progenitors CD31 to compare the amount of blood vessels. In the ovaries of Decapeptyl-treated mice, IF staining score for CD31 was significantly lower than in controls (6.36 ± 0.6 vs 17.32 ± 0.5, p=0.0001****) (**Figure 9**).

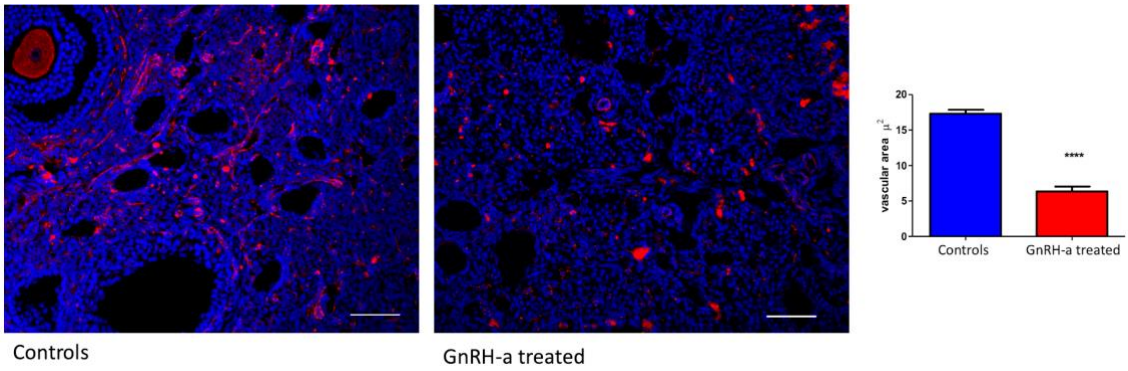

Figure 9. *Immunofluorescence images of the ovary of an untreated and a GnRH-a treated mouse showing endothelial cells stained with CD31 (red) and cell nuclei with 4',6-diamidino-2-phenylindole (DAPI) (blue). In treated mouse, regression of small vessels can be observed. Quantitative analysis confirms a significant decrease in CD31 positive area per square microns in GnRH-a treated mice compared to controls. Scale bar 20x, 50 µm.*

5.2 GnRH-a-induced reduction of ovarian perfusion could be protective over alkylating-agent mediated gonadotoxicity

In the second part of the experiment, 12 mice were treated simultaneously with an intraperitoneal injection of cyclophosphamide 75 mg/kg and 12 mice were treated with an intraperitoneal injection of doxorubicin 2 mg/kg. GnRH-a was administered at day -14 and repeated at day +14 from chemotherapy. For the whole duration of this experiment, mice were globally healthy and showed good tolerance to treatments.

US assessments

Similarly to the previously described study, the diameter of the dominant follicle increased significantly in mice treated with decapeptyl compared to those treated with saline solution, at 14 days after its administration. The effect on the dominant follicle diameter was observed independently from chemotherapy administration. At timepoint IV, mice receiving cyclophosphamide and decapeptyl showed higher dominant follicle diameters compared to those only receiving decapeptyl (average follicle diameter in mice treated with cyclophosphamide and decapeptyl vs average follicle diameter in mice

treated with decapeptyl, 0.563 ± 0.122 mm vs 0.385 ± 0.155 mm, *p=0.01). This increase of folliculogenesis was less pronounced in the group treated with doxorubicin, as shown in **Figure 10**.

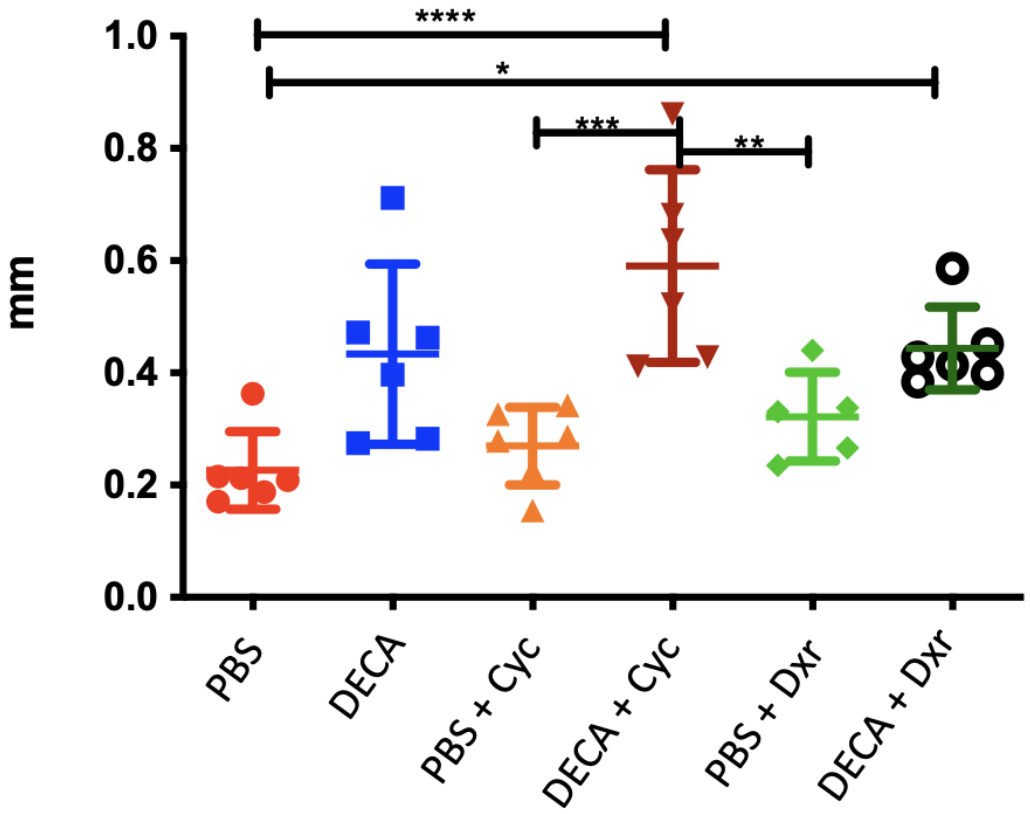

Figure 10. *Dominant follicle diameters (mm) at Timepoint IV (Bars: mean ± SD). PBS: mice treated with saline solution (n=12, left and right ovary); DECA: mice treated with decapeptyl (n=12, left and right ovary); PBS+Cyc: mice treated with saline solution and cyclophosphamide (n=12, left and right ovary); DECA+Cyc: mice treated with decapeptyl and cyclophosphamide (n=12, left and right ovary); PBS + Dxr: mice treated with saline solution and doxorubicin (n=12, left and right ovary); DECA+Dxr: mice treated with decapeptyl and doxorubicin (n=12, left and right ovary).*

CEUS assessments

For this study, the 2 most important perfusion parameters were PE (expression of the width of the vascular bed) and WiR (expression of the velocity of blood flow).

Mice treated with cyclophosphamide showed a statistically significant increase in PE at timepoint IV compared with controls treated with saline solution. Administration of decapeptyl seems to decrease this effect, as mice treated with decapeptyl and

cyclophosphamide showed reduced PE at timepoint IV compared to mice treated with cyclophosphamide and saline solution.

Similarly, administration of cyclophosphamide increased WiR (blood velocity) and AUC (blood quantity in the vascular system) at timepoint IV, whereas concomitant administration of decapeptyl reduced this effect, keeping WiR and AUC at timepoint IV lower. Conversely, mean transit time (mTT) was reduced in mice treated with cyclophosphamide with respect to controls (**Figure 11, Figure 12, Figure 13**).

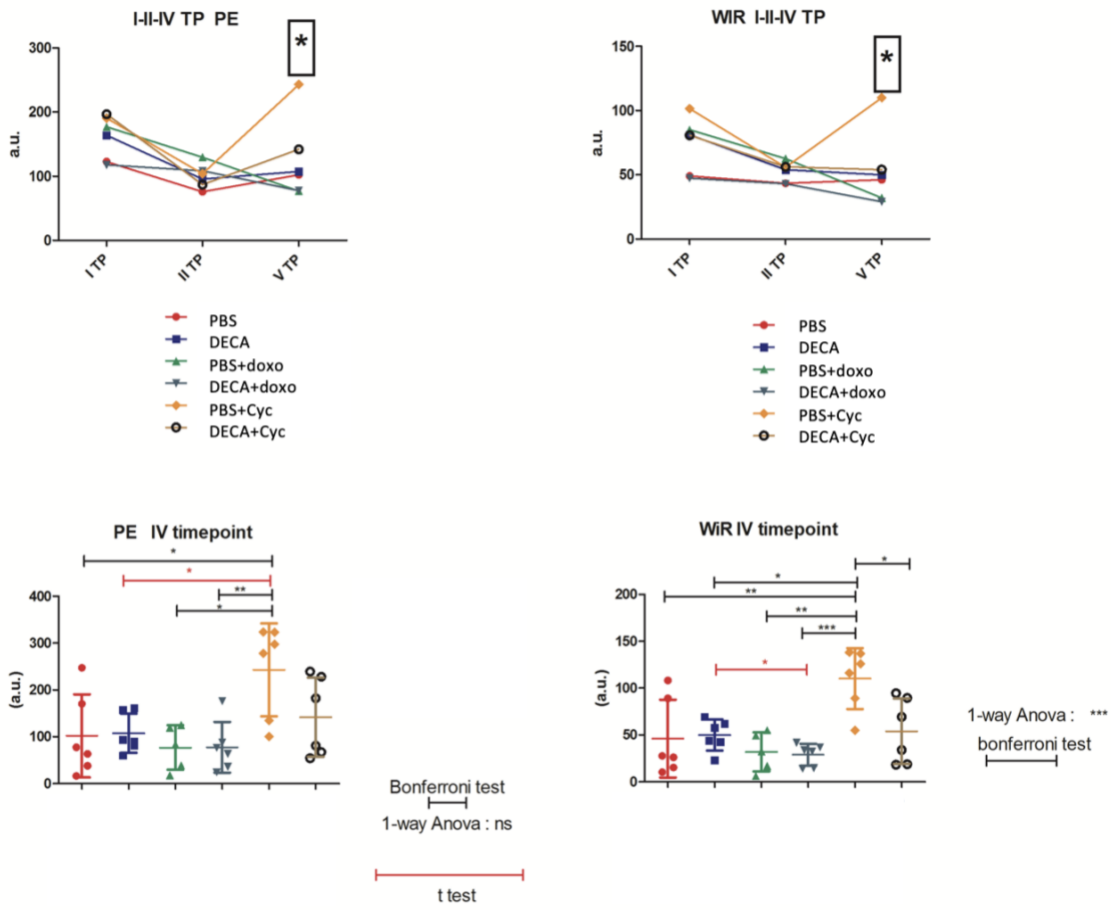

Figure 11. *Top, left: Peak Enhancement (PE) at timepoints I, II and IV: a significant increase in PE can be observed in mice treated with PBS+Cyclophosphamide. Top, left: Wash-in-rate (WiR) at timepoints I, II and IV: a significant increase in WiR can be observed in mice treated with PBS+Cyclophosphamide. Bottom, left: PE at timepoint IV in all groups. Bottom, right: WiR at timepoint IV in all groups.*

PBS: mice treated with saline solution (n=12, left and right ovary); DECA: mice treated with decapeptyl (n=12, left and right ovary); PBS+Cyc: mice treated with saline solution and cyclophosphamide (n=12, left and right ovary); DECA+Cyc: mice treated with decapeptyl and

cyclophosphamide (n=12, left and right ovary); PBS + Dxr: mice treated with saline solution and doxorubicin (n=12, left and right ovary); DECA+Dxr: mice treated with decapeptyl and doxorubicin (n=12, left and right ovary).

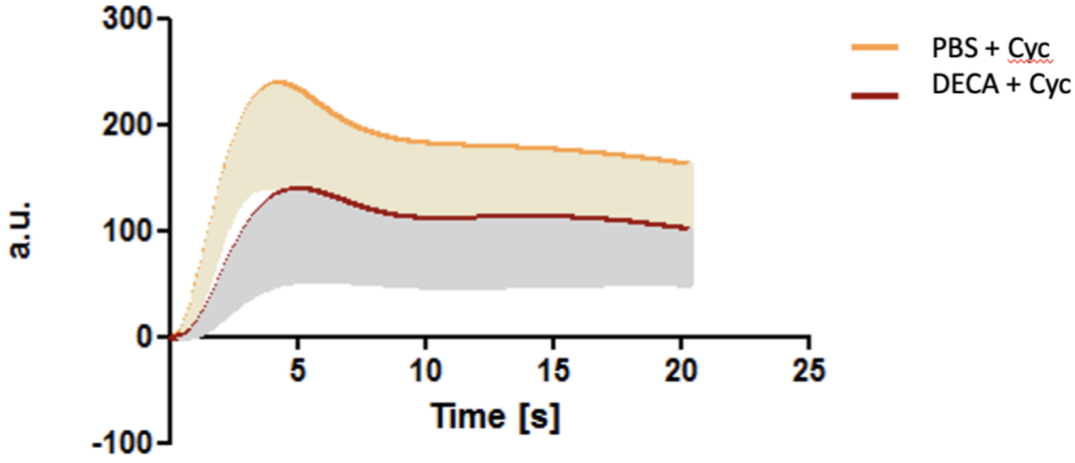

Figure 12. *Perfusion curve of the PBS+Cyclophosphamide group vs Decapeptyl+Cyclophosphamide.*

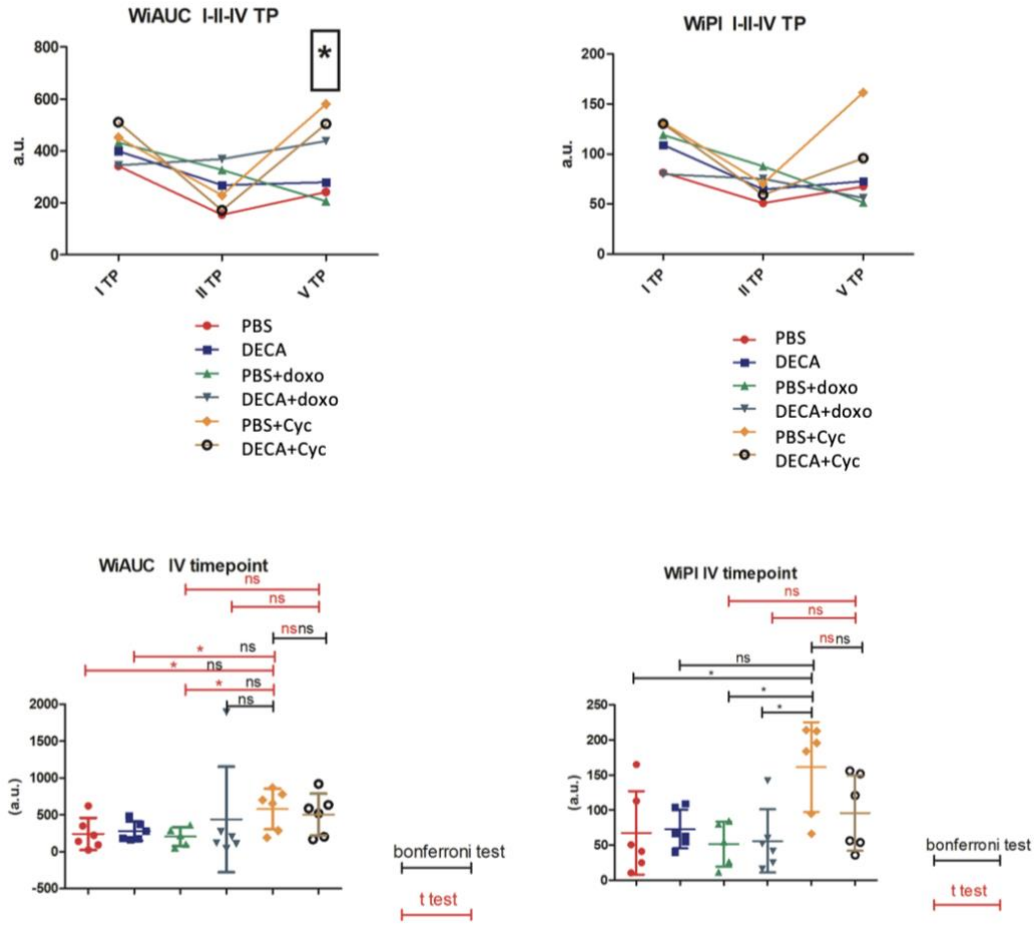

Figure 13. *Top, left: WiAUC (Wash-in area under the curve) at timepoints I, II and IV: a significant increase in WiAUC can be observed in mice treated with PBS+Cyclophosphamide. Top, left: WiPI (Wash-in perfusion index) at timepoints I, II and IV: a significant increase in WiPI can be observed in mice treated with PBS+Cyclophosphamide. Bottom, left: WiAUC at timepoint IV in all groups. Bottom, right: WiPI at timepoint IV in all groups.*

PBS: mice treated with saline solution (n=12, left and right ovary); DECA: mice treated with decapeptyl (n=12, left and right ovary); PBS+Cyc: mice treated with saline solution and cyclophosphamide (n=12, left and right ovary); DECA+Cyc: mice treated with decapeptyl and cyclophosphamide (n=12, left and right ovary); PBS + Dxr: mice treated with saline solution and doxorubicin (n=12, left and right ovary); DECA+Dxr: mice treated with decapeptyl and doxorubicin (n=12, left and right ovary).

5.3 No difference in ovarian perfusion after administration of concurrent GnRH-a and doxorubicin vs doxorubicin alone

The group of mice treated with doxorubicin showed less changes in perfusion parameters compared with controls treated with saline solution. We could observe an

increase of mTT in all mice treated with doxorubicin, with respect to mice treated with cyclophosphamide and mice treated with decapeptyl alone. Adding decapeptyl to doxorubicin did not significantly alter perfusion parameters.

5.4 Studies on human ovarian tissue

5.4.1 Can OTC be performed after the beginning of chemotherapy?

The purpose of this analysis was the evaluation of the adequacy of performing OTC in young post-pubertal women who already started a gonadotoxic treatment. In fact, for some patients this was the last opportunity to safely retrieve ovarian tissue before a consistent damage to the follicular reserve occurred. The actual validity of the procedure is currently unknown, due to paucity of literature data on follicular viability and pregnancy rates after re-transplantation.

The results of this analysis have been recently published (Cioffi, Cervini, et al., 2022).

Data was collected in a prospective fashion to gather information about all OTC procedures performed at our Unit between 2012 and 2020, which in total were 79. We decided to compare histological follicular counts from ovarian tissue biopsies of patients undergoing the procedure according to whether they already received chemotherapy or not. We also compared pre-procedural AMH serum levels, collected the day before the OTC laparoscopy.

For this analysis, we decided to focus on patients older than 15 years, because we thought that younger patients were worthy of a separate evaluation due to differences in

ovarian physiology. We also excluded patients with a pre-existing ovarian condition such as endometriosis.

The study included 30 patients, of which half had undergone a gonadotoxic treatment before the procedure (Group 1) while the other half was treatment-naïve (Group 2). Most of the patients had received a high or intermediate gonadotoxicity schedule. Time elapsed between last chemotherapy dose and procedure varied, but in most cases (66% of patients) OTC was carried out no later than a month after the last chemotherapy course.

Age was comparable between the 2 groups (**Table 4**).

	Total population	Group 1	Group 2	P-value
Age at procedure (years, median, mean ± standard deviation [range])	18, 20.63 ± 6.49 (15-38)	18, 20.07 ± 5.74 (15-36)	18, 21.20 ± 7.33 (15-38)	ns
AMH (ug/L, median, mean ± standard deviation [range])	1.7, 2.32 ± 2.42 (0.02-10.20)	0.74, 2.14 ± 3.02 (0.02-10.20)	1.8, 2.54 ± 1.55 (0.94-5.08)	ns

Table 4. Patients' age and pre-procedural serum anti-Mullerian hormone (AMH) levels. Group 1: patients who received chemotherapy; Group 2: patients who did not receive chemotherapy (from (Cioffi, Cervini, et al., 2022) according to fair use principle)

Patients' diagnoses and details about chemotherapy schedules and number of pre-OTC cycles are reported in **Table 5**.

Pathology	Patients exposed to chemotherapy (N)	Protocols or drugs used	Mean number of pre-OTC cycles (range)
Sarcoma (N=6)	2	Dox-Ifos	1.5 (1-2)
	2	IVADo	3 (2-4)
	1	IVA	9 (9)
	1	CAV	1 (1)
Gynecological cancer (N=1)	1	PC	2 (2)
Lymphoma (N=4)	4	ABVD	3.8 (1-6)
Germ cell tumor (N=2)	2	BEP	3 (2-4)
Other tumor (N=1)	1	VAD	2 (2)
Multiple sclerosis (N=1)	1	Cyclophosphamide	7 (7)

ABVD = adriamycin/doxorubicin, bleomycin, vinblastine, dacarbazine; BEP = bleomycin, etoposide, platinum; CAV = cyclophosphamide, adriamycin/doxorubicin, vincristine; Dox-Ifos = doxorubicin, ifosfamide; IVA = ifosfamide, vincristine, actinomycin-D; IVADo = ifosfamide, vincristine, actinomycin-D, doxorubicin; PC = paclitaxel, carboplatin; VAD = vincristine, actinomycin-D, doxorubicin

Table 5. Diagnoses and pre-procedural chemotherapeutic protocols in Group 1 patients (n=15) (from (Cioffi, Cervini, et al., 2022) according to fair use principle)

Table 6 reports the results of the principal analysis of the study, i.e. the comparison between AMH serum levels and histological follicular counts divided by subtype between treated and untreated women.

	Group 1 (n=15)	Group 2 (n=15)	Age adjusted p-value
AMH (ug/L, median; mean)	0.74; 2.14	1.8; 2.54	0.70
Primordial follicles (N, median; mean)	18; 34.67	23; 38.21	0.73
Primary follicles (N, mean)	14; 14.94	8.5; 9.53	**0.04**
Early secondary follicles (N, median; mean)	0; 0.35	0; 0.62	0.52
Late secondary follicles (N, median; mean)	0; 0.07	0; 0.27	0.26
Atretic follicles (N, median; mean)	2; 4.92	1.25; 2.05	**0.05**
Dysfunctional follicles (N, median; mean)	0; 0.10	0; 0.14	0.77
Total functional follicles (N in 10 areas of 1 mm^2, median; mean)	63; 50.01	63; 48.63	0.94
Total functional follicles (N in 1 mm^3, median; mean)	144; 100.02	144; 97.25	0.90

Table 6. Anti-Mullerian hormone (AMH) levels and follicular counts in patients receiving pre-cryopreservation chemotherapy (Group 1) or no chemotherapy (Group 2) (from (Cioffi, Cervini, et al., 2022) according to fair use principle)

In summary, we found no significant difference between AMH levels and primordial follicle counts, suggesting that after 1 or 2 cycles of chemotherapy, ovarian reserve appeared still grossly intact. As expected, chemotherapy increases the number of atretic follicles (2 vs 1.25, p=0.05). We also noticed, interestingly, that primary follicles were significantly higher in those receiving chemotherapy (14 vs 8.5, p=0.04), as if a greater number of dormant follicles were activated by the gonadotoxic insult and resumed meiosis.

5.5 Clinical studies

5.5.1 Prognostic factors for suboptimal response to COS in breast cancer patients

The objective of this analysis was the identification of the impact of cancer stage, cancer grade and immunohistochemical prognostic factors on the outcomes of oocyte cryopreservation in patients referring to our Oncofertility Unit from 2011 to 2019 for a breast cancer diagnosis.

The results of this part of the project have been published at the end of the second year of the PhD Program (Cioffi et al., 2021).

Figure 14 shows the process of patient selection for this retrospective analysis.

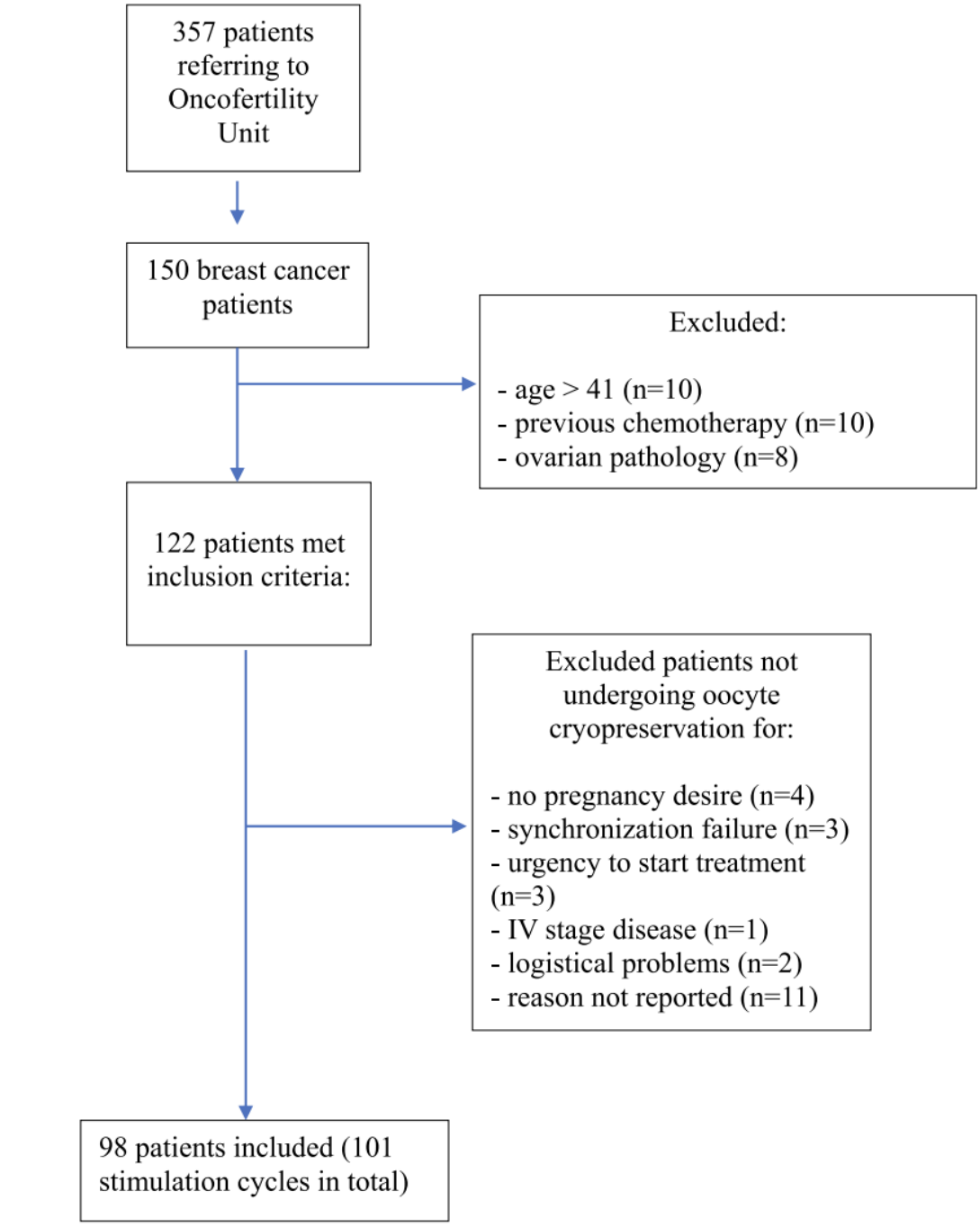

Figure 14. *Flow-chart of patient selection (from Cioffi et al., 2021, according to fair use principle).*

Analyses according to breast cancer stage

This analysis was conducted on 72 patients in total.

We classified patients according to the 2017 edition of American Joint Committee on Cancer (AJCC): stage I patients were considered as low-stage breast cancer, while stage II-III were considered as high-stage breast cancer.

Table 7 and **Table 8** report the results of this analysis.

	Low-stage disease	High-stage disease	p-value	Age-adjusted p-value
Number of patients	36	36		
Age (years)	38 (31–41)	33 (26–40)	**0.004**	
BMI (kg/m^2)	20.1 (17–34)	20.6 (18–28)	0.93	0.60
Smoking [n (%)]	4 (11)	4 (11)	0.83	0.23
BRCA mutation [n (%)]	6 (16)	12 (33)	0.35	0.64
AFC (n)	12 (5–28)	10 (2–25)	0.92	0.46
AMH (ug/L)	1.9 (0.2–10.6)	1.8 (0.4–12.5)	0.22	0.89

BRCA: Breast-related Cancer Antigens.
AFC: Antral Follicle Count.
AMH: Anti-Mullerian Hormone.
All values are median.

Table 7. *Characteristics of patients according to breast cancer stage. (from Cioffi et al., 2021, according to fair use principle).*

Patients with high-stage cancer were younger than patients affected by a low-stage cancer. The results of all the univariate analyses were therefore corrected for the effect of age.

Basal AFC and AMH were not significantly different between low-stage and high-stage disease patients.

	Low-stage disease	High-stage disease	p-value	Age-adjusted p-value
FSH start dose (IU)	225 (125–300)	225 (112–450)	0.62	0.29
Days of stimulation	11 (6–15)	11 (7–15)	0.92	0.83
Total FSH dose (IU)	2525 (1400–4050)	2400 (1200–4500)	0.80	0.66
Estradiol on trigger day (pg/mL)	468 (63–4706)	621 (63–4727)	0.28	0.58
Follicles>16 mm on trigger day (n)	8 (2–47)	6 (1–21)	0.62	0.11
Oocytes (n):				
Retrieved	9 (1–34)	11 (2–40)	0.18	0.69
MII vitrified	7 (1–25)	7 (1–24)	0.34	0.75
Maturation rate (mean%)	75	73	0.59	0.89

MII: metaphase II.
All values are median.

Table 8. *Controlled ovarian hyperstimulation protocols and outcomes according to cancer stage (from Cioffi et al., 2021, according to fair use principle).*

We found no significant differences in the gonadotropin doses used during stimulation and the number of oocytes retrieved between high-stage and low-stage cancer patients.

Analyses according to breast cancer grade

This analysis was conducted on 84 patients in total.

The low-grade breast cancer group includes patients with a G1-G2 disease, while the high-grade breast cancer group includes patients with a G3 disease.

Table 9 and **Table 10** report the results of this analysis.

	Low-grade disease	High-grade disease	p-value	Age-adjusted p-value
Number of patients	45	39		
Age (years)	36 (29–41)	33 (26–39)	0.60	
BMI (kg/m^2)	20 (17–34)	20 (18–28)	0.31	0.62
Smoking [n (%)]	5 (11)	4 (10)	0.91	0.67
BRCA mutation [n (%)]	4 (9)	17 (43)	0.001	0.001
AFC (n)	13 (5–28)	10 (2–25)	0.14	0.03
AMH (ug/L)	2.1 (0.4–10.6)	1.5 (0.2–12.5)	0.88	0.47

BRCA: Breast-related Cancer Antigens.
AFC: Antral Follicle Count.
AMH: Anti-Mullerian Hormone.
All values are median.

Table 9. *Characteristics of patients according to breast cancer grade (from Cioffi et al., 2021, according to fair use principle).*

We found a significant association in our cohort between BRCA mutation and occurrence of a high-grade cancer.

With respect to basal fertility indicators, we noticed an interesting difference in age-adjusted antral follicular count (AFC), that was significantly lower in high-grade breast cancer patients compared with patients with a low-grade disease (p=0.03).

	Low-grade disease	High-grade disease	p-value	Age-adjusted p-value
FSH start dose (IU)	2250 (1200–3750)	2612 (1200–4500)	0.07	0.03
Days of stimulation	225 (100–300)	225 (112–450)	0.33	0.06
Total FSH dose (IU)	10 (6–15)	11 (7–14)	0.67	0.77
Estradiol on trigger day (pg/mL)	454 (83–4706)	612 (63–4727)	0.52	0.97
Follicles>16 mm on trigger day (n)	7 (1–47)	6 (1–22)	0.67	0.29
Oocytes (n):				
Retrieved	9 (1–28)	9 (2–24)	0.42	0.67
MII vitrified	6 (1–21)	7 (1–24)	0.22	0.35
Maturation rate (mean%)	75	80	0.43	0.25

MII: metaphase II.
All values are median.

Table 10. *Controlled ovarian hyperstimulation protocols and outcomes according to cancer stage (from Cioffi et al., 2021, according to fair use principle).*

As with the analyses performed for cancer stage, oocyte retrieval did not significantly differ between high-grade and low-grade disease patients. However, we noticed that a significantly higher dose of FSH was required during ovarian stimulation in patients with a high-grade disease, compared to those with low-grade cancer (p=0.03).

5.5.2 Controlled ovarian stimulation outcomes in patients with a sarcoma

The objective of this analysis was to compare basal fertility and outcomes of COS of a population of women with a diagnosis of sarcoma with those of a control population of women without cancer.

For this retrospective analysis, a total of 63 women with a diagnosis of sarcoma were included, of which 43 underwent COS at our Unit between 2013 and 2021.

The control population included 355 patients undergoing COS for isolated severe male factor infertility, that we defined as fewer than 0,15 million motile sperm per milliliter of semen. Women with endometriosis, idiopathic infertility and tubal disease were excluded.

Sarcoma cases were matched with controls of comparable age (range ± 1.5 years) and year of stimulation (range ± 1.5 years). For women under 32 years of age, due to a lower average age in the sarcoma group with respect to the control group, age was set to a standard value of 29 for the purpose of the matching.

In the final analysis, a total of 37 patients with sarcoma were matched in a 1:3 ratio with 109 healthy controls.

Primary study outcomes included pre-treatment AFC and number of retrieved mature oocytes after stimulation. Secondary study outcomes included total dose of gonadotropin administered, days of stimulation, and FSH/oocyte ratio.

Table 11, **12** and **13** report the results of univariate comparisons between sarcoma cases and controls respectively in the whole population, in the population of women younger than 32 years and in the population of women older than 32 years.

	Sarcoma (N=37)	Controls (N=109)	p-value
Age (years)	29.4±5.9	32.8± 3.5	**0.001**
BMI (kg/m^2)	21.4±3.1	21.4±3.2	0.975
AFC (n)	13.2±6.9	12.7±6.1	0.670
Days of stimulation (n)	10.3±2.0	9.7±1.9	0.155
FSH total dose (IU)	1643.7±1168.0	2001.1±932.0	**0.007**
Estradiol on trigger day (pg/ml)	1331.9±835.9	2078.1±1147.5	**0.0001**

Progesterone on trigger day (ng/ml)	0.8±1.0	0.7±1.2	0.534
Retrieved oocytes (n)	13.0±8.3	10.3±5.8	0.147
MII oocytes (n)	10.6±7.1	8.1±5.2	0.064
FSH/oocyte ratio	198.4±200.7	326.8±389.4	**0.009**

Table 11. *Characteristics of patients with a sarcoma and healthy controls (whole population). All the results are expressed as mean ± standard deviation.*

	Sarcoma (N=18)	Controls (N=52)	p-value
Age (years)	24.2±2.9	30.9±2.3	**0.0001**
BMI (kg/m^2)	21.2±2.9	22.0±3.0	0.294
AFC (n)	14.7±7.9	14.3±6.7	0.819
Days of stimulation (n)	9.6±2.0	9.7±1.7	0.594
FSH total dose (IU)	1510.7±1359.0	1665.4±1205.8	0.120
Estradiol on trigger day (pg/ml)	1525.4±1035.3	2127.7±1274.3	0.054
Progesterone on trigger day (ng/ml)	0.8±1.2	0.7±1.6	0.329
Retrieved oocytes (n)	16.3±8.9	10.8±6.2	**0.011**
MII oocytes (n)	13.0±7.6	8.6±5.7	**0.009**
FSH/oocyte ratio	126.4±157.7	282.4±411.2	**0.005**

Table 12. *Characteristics of patients with a sarcoma and healthy controls (women ≤ 32 years of age). All the results are expressed as mean ± standard deviation.*

	Sarcoma (N=19)	Controls (N=57)	p-value
Age (years)	34.3±3.1	34.6±3.5	0.600

BMI (kg/m^2)	21.7±3.4	20.9±3.2	0.355
AFC (n)	11.7±5.7	11.1±5.2	0.660
Days of stimulation (n)	10.8±1.9	9.7±2.0	**0.022**
FSH total dose (IU)	1769.7±974.6	2307.3±1005.9	**0.017**
Estradiol on trigger day (pg/ml)	1138.4±543.6	2032.9±1100.8	**0.001**
Progesterone on trigger day (ng/ml)	0.7±0.9	0.6±0.7	0.975
Retrieved oocytes (n)	9.8±6.6	9.8±5.4	0.691
MII oocytes (n)	8.3±5.9	7.7±4.6	0.909
FSH/oocyte ratio	262.8±216.7	367.3±367.4	0.210

Table 13. *Characteristics of patients with a sarcoma and healthy controls (women over 32 years of age). All the results are expressed as mean ± standard deviation.*

Considering the whole population, patients diagnosed with a sarcoma were significantly younger than controls. BMI was comparable between the 2 populations.

In the univariate analysis, average AFC did not significantly differ between patients with a sarcoma and their age-matched controls (13.2±6.9 vs 12.7±6.1, p-value= ns). Significant differences could be observed between the stimulation protocols of sarcoma patients and controls: in particular, lower FSH doses were used in patients undergoing cryopreservation for a sarcoma, compared with patients undergoing the procedure for ART (1643.7±1168.0 vs 2001.1±932.0, p=0.007). This resulted in different hormone levels on trigger day, with a trend for higher levels of estradiol in the control group.

In general, patients with a sarcoma did not perform worse than controls during stimulation, with an average MII retrieval of 10.6 oocytes vs 8.1 in the control population. Interestingly, they also showed a more favorable FSH/oocyte ratio (198.4±200.7 vs 326.8±389.4, p=0.009).

In the subgroup analysis, women ≤32 years of age had a less precise matching due to the higher average age in the control group. This partially explains a higher number of retrieved oocytes in the sarcoma group, composed of younger patients, with respect to controls. Over 32 years of age, instead, where the matching was very accurate, no differences in oocyte retrieval outcomes were observed between sarcoma patients and

controls. In this group, the difference in stimulation protocols is more evident, with higher FSH doses and higher levels of estradiol in the control group.

In the multivariate analysis, a linear regression was performed on the outcome of number of retrieved MII oocytes. Factors included in the regression were sarcoma diagnosis, AFC and age. **Table 14** reports the results in the whole population and in the two subgroups of women under age 32 and over age 32.

	Coefficient (estimate of MII difference in the group with a sarcoma)*	IC 95%	p-value
Whole population	1.206	-0.824 – 3.235	0.242
Age ≤ 32 years	2.720	-2.122 – 7.562	0.266
Age > 32 years	0.371	-1.994 – 2.736	0.755

Table 14. *Results of linear regression analysis of number of MII oocytes in patients with a sarcoma and controls corrected by age and AFC in the whole population and in the 2 age subgroups (below and over 32 years).*

* Corrected by age and AFC.

Results of linear regression on the number of retrieved mature oocytes confirmed the non-inferior performance of the group of patients with a sarcoma during COS.

5.5.3 Development of a predictive model for premature ovarian insufficiency in young women undergoing chemotherapy

We retrospectively analyzed the rate of chemotherapy-induced amenorrhea, POF and infertility among patients referring to our Oncofertility Unit for counselling and oocyte cryopreservation.

The results of this part of the project have been published in a recent paper (Cioffi, Fais, et al., 2022).

Study population included 348 patients receiving counselling from August 2011 to January 2020.

Follow-up data on menstrual and reproductive function could be obtained for a total of 184 patients, after excluding patients that were dead, lost at follow-up or receiving hormonal therapy at the time of contact.

Figure 15 shows the distribution of cancer types in the population.

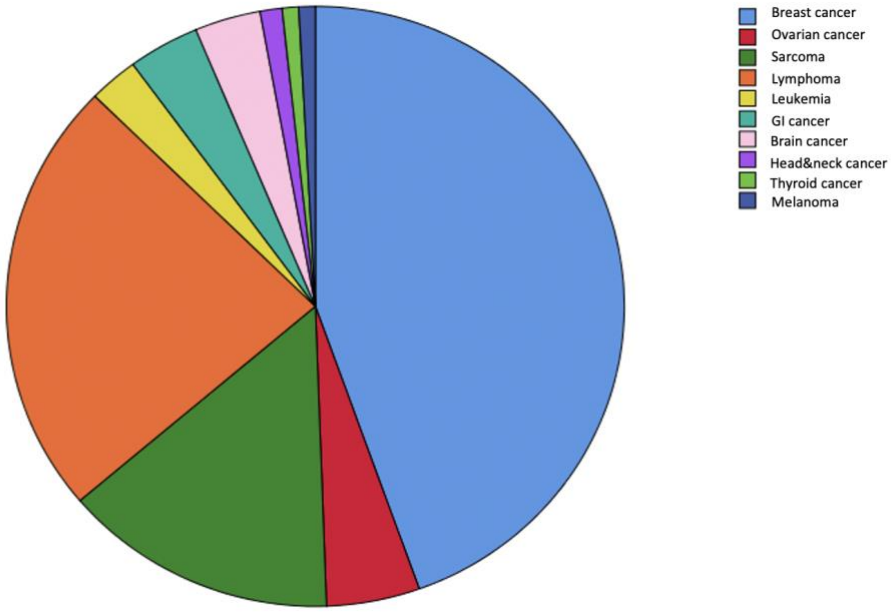

Figure 15. *Distribution of cancer types in study population. GI: gastrointestinal.*

The POF rate in our population was 24.5%, while the infertility rate was 32.6%.

Table 15 reports the factors that were associated with a higher prevalence of POF in univariate analysis.

Factor	OF	No OF	p-value
Age	31.0 ± 6.0	30.1 ± 6.5	ns
BMI	21.0 ± 2.3	21.4 ± 3.1	ns
AMH	1.2 ± 1.1	2.2 ± 1.8	0.03
AFC	10.3 ± 6.4	12.2 ± 6.7	ns
FSH	10.2 ± 7.1	7.4 ± 3.0	0.01
Leukemia diagnosis	4/7 (57.1%)	3/7 (42.9%)	0.06
High-stage cancer	15/39 (38.5%)	24/39 (61.5%)	0.03
Chemotherapy lines (n)	1.9 ± 0.9	0.9 ± 0.6	0.002

Data are expressed as mean ± standard deviation or percentages.
AFC: Antral follicle count; AMH: Anti-Müllerian hormone; FSH: Follicle-stimulating hormone; ns: Not significant; OF: Ovarian failure.

Table 15. *Factors associated with a higher probability of POF in our population (from Cioffi et al., 2022, according to fair use principle).*

Table 16 shows chemotherapy schedules in our population and relative odd-ratios for POF.

Schedule	OF prevalence (%)	OR (95% CI)	p-value
Platinum-based	8/24 (33)	1.6 (0.6–4)	ns
TaxAC-CMF	7/18 (39)	2.0 (0.7–5.7)	ns
VAI/AI	2/8 (25)	1 (0.1–5.1)	ns
EPI-IFO	9/19 (47)	3.1 (1.1–8.2)	0.02
Capecitabine	3/4 (75)	9.5 (0.9–94.4)	0.04
ABVD	2/41 (4.9)	0.1 (0.02–0.4)	0.0001
CHOP	8/13 (62)	5.6 (1.7–18.2)	0.004
FEC + weekly paclitaxel	3/16 (19)	0.6 (0.1–2.4)	ns
Oncocarbide	0/4 (0)	0.7 (0.6–0.8)	ns
Fludarabine	1/3 (33)	1.5 (0.1–17.0)	ns
BEACOPP	3/5 (60)	4.7 (0.7–29.3)	0.09
Temozolomide	2/7 (29)	1.2 (0.2–6.4)	ns
Monoclonal antibody	8/24 (33)	1.6 (0.6–4)	ns
Trabectedin	1/5 (20)	0.7 (0.08–6.8)	ns
High-dose	22/24 (94)	63.6 (14–289)	0.0001

ABVD: Adriamycin, bleomycine, vinblastine and doxorubicin; BEACOPP: Bleomycin, etoposide, adriamycin, cyclophosphamide, vincristine, procarbazine and prednisone; CHOP: Cyclophosphamide, doxorubicin, vincristine and prednisone; EPI-IFO: Epirubicin and ifosphamide; FEC: Fluorouracil, epirubicine and cyclophosphamide; ns: Not significant; OF: Ovarian failure; OR: Odds ratio; TaxAC-CMF: Anthracycline and taxane–cyclophosphamide, methotrexate, and fluorouracil; VAI/AI: Vincristine, adriamycin and ifosphamide/adriamycin and ifosphamide.

Table 16. *Results of chi-square tests of chemotherapy regimens and POF in our population (from Cioffi et al., 2022, according to fair use principle).*

Using logistic regression analysis, the best model that described the population undergoing POF was selected. It included a combination of 5 factors as shown in **Table 17**.

Factor	Odds Ratio	95% CI	p-value
Age	1.0	0.9–1.1	0.3
Chemotherapy lines (n)	10.5	4.6–23.9	0.0001
VAI/AI	86.8	3.7–2029.3	0.005
Capecitabine	0.06	0.006–0.6	0.02
ABVD	15.8	1.9–126.2	0.009

ABVD: Adriamycin, bleomycine, vinblastine and doxorubicin; VAI/AI: Vincristine, adriamycin and ifosphamide/adriamycin and ifosphamide.

Table 17. *Logistic regression analysis with the factors entering the predictive model for POF after chemotherapy.*

Propensity scores for POF were calculated for each patient in the analysis and the resulting receiver operating curve (ROC) curve for the model is shown in **Figure 16**.

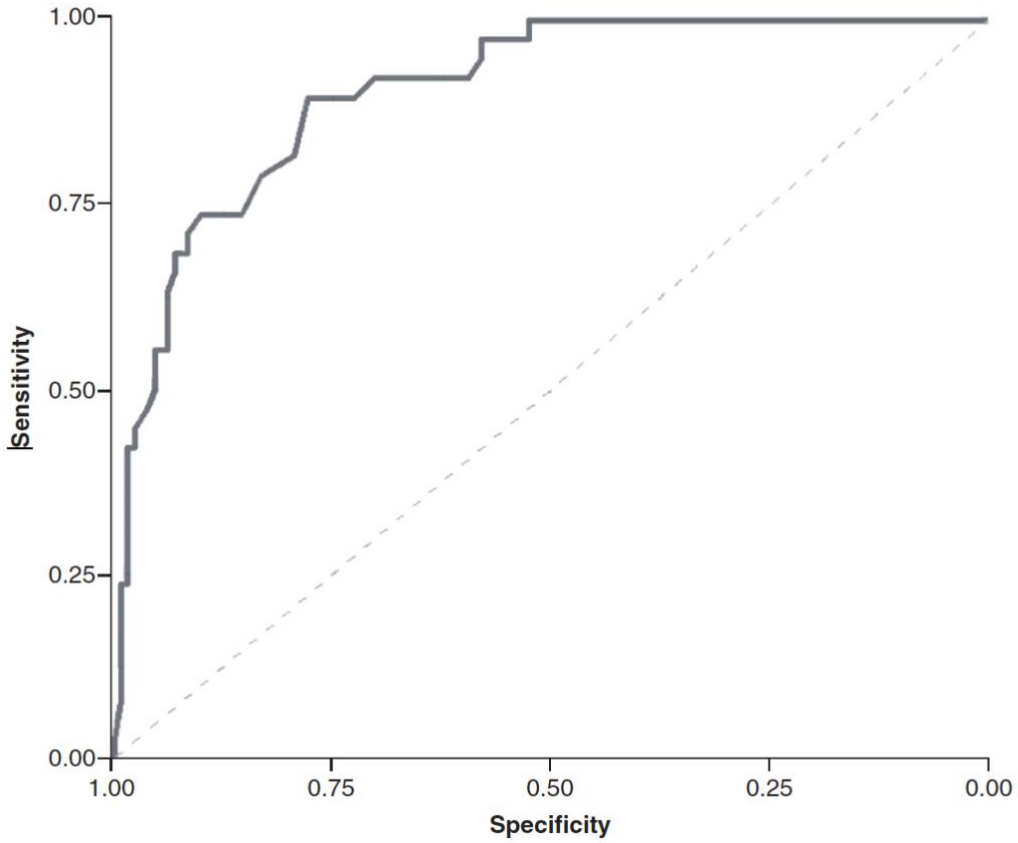

Figure 16. *Receiver operating curve for POF probability calculated combining age, number of chemotherapy lines, VAI/AI, capecitabine and ABVD. AUC = 0.906 (0.858–0.954 CI 95%), p = 0.0001 (from Cioffi et al., 2022, according to fair use principle).*

5.5.4 An ultrasound protocol of chemotherapy-related uterine damage

Objectives

Primary aim of this study is the identification of uterine ultrasound changes on measurements, shape, structure, and blood supply after exposure to systemic

chemotherapy. In case of positive findings on the primary outcome, secondary aims involve longitudinal assessment of these effects overtime to ascertain whether they are permanent or reversible and correlate them to patients' pregnancy outcome.

Study design and population

We designed a monocentric, prospective, observational, cohort study of patients with diagnosis of cancer, with nested analysis of exposure and non-exposure to systemic chemotherapy.

Patients referring to IRCCS San Raffaele Hospital Oncofertility Unit (Milan, Italy) for counselling on fertility preservation procedures will be consecutively enrolled. All patients will sign a written informed consent prior to enrollment into the study.

The inclusion criteria will be: a) post-menarchal patients; b) age 18-43 years; c) recent cancer diagnosis, with or without indication to chemotherapeutic treatment. Patients not receiving chemotherapy will be included in the analysis as controls.

The exclusion criteria will be: a) pre-menarchal patients; b) previous uterine and/or adnexal surgery; c) denial of informed consent.

Materials and methods

For the purpose of this study, ultrasound assessments will be performed in all patients at the following timepoints: basal assessment (before the beginning of chemotherapy); between 1 month and 6 months by the end of chemotherapy, and every 12 months thereafter.

A database of patients' characteristics will be filled out including all demographic, biometric characteristics of each patient (weight, height, body mass index [BMI]), phase of menstrual cycle, associated pre-existing medical conditions, type of cancer and staging, surgical treatments performed, medical treatment administered (e.g. hormonal or immunological treatments). Timing and extent of chemotherapy will be recorded including number, type of drug and dose of each medication used.

Grey-scale Ultrasound protocol

Ultrasound evaluations will be carried out by a 4-9 MHz transvaginal probe using dedicated machine presets for gynecology. The ultrasound beam frequency will be set at an average level of 7 MHz, with a frequency range of 1000-5000 Hz. Doppler gain will be set at the lowest level (range 40–60%) allowing recording of adequate signals avoiding noise and a wall filter of 100 Hz will be used. Measurements will be standardized.

The following parameters will be evaluated and subsequently analyzed: uterine dimensions and volume; endometrial thickness; myometrial thickness, cervix-corpus ratio; ovarian dimensions, follicular count.

Elastosonography protocol

Strain elastography will be applied by using a digital ultrasound device (Samsung, HeraW10), including real-time elastography software. Bidimensional shear wave elastography will be performed using the same setting and frequency range of the B-mode ultrasound examination. Elastograms will be displayed in real time. Evaluation of tissue stiffness will be performed by selecting a region of interest (ROI) with an adjustable diameter up to 10 mm and obtaining a color scale of the elastic modulus in kilopascals (kPa). The minimum and maximum stiffness and the mean elasticity values will be measured separately for each ROI (Manchanda et al., 2019).

The following parameters will be evaluated and compared: mean elasticity of endometrium; mean elasticity of myometrium; mean elasticity of cervix; endometrial-to-myometrial elasticity ratio (E/M ratio).

Doppler ultrasound protocol

Spectral pulsed Doppler of uterine arteries will be performed transvaginally according to standard procedure (Papageorghiou et al., 2001). The following indices will be calculated and subsequently analyzed: Resistance Index (RI); Pulsatility Index (PI); Peak Systolic Velocity (PSV); Time Averaged Maximum Velocity (TAMax).

Preliminary results of the study

During the PhD project, the first 54 patients were consecutively enrolled into this study. Twelve patients came back to the clinic for the second scan. Only grey-scale and doppler evaluations were performed in these patients, due to unavailability of strain elastography.

Patients' mean age was 32 years (range 19-42). Twenty-four patients (44%) had breast cancer, 15 patients (28%) had a lymphoma, 11 patients (20%) had a sarcoma, 3 patients (6%) had brain cancer, 1 patient (2%) had a gastrointestinal cancer.

For future analyses, patients will be stratified according to menstrual phase (proliferative vs secretory phase), determined on the basis of last menstrual period, endometrial thickness and presence of corpus luteum, and according to type of chemotherapy.

Preliminary analyses showed a significantly reduced uterine volume pre vs post-chemotherapy (median volume 40.2 cm^3 vs 29 cm^3, p=0.04). However, when excluding patients in treatment with oral contraceptives and GnRH-a, uterine volume appeared comparable between the 2 groups (40.2 cm^3 vs 41.8 cm^3, N=47 vs N=6, p=0.7). No significant difference could be observed in the median longitudinal, anteroposterior, transverse, or cervical diameters. We decided to exclude from further analyses patients in treatment with oral contraceptives and GnRH-a.

When comparing doppler parameters, previa exclusion of patients in treatment with GnRH and oral contraceptives, a significant increase in median RI could be observed pre- to post-chemotherapy (0.83 vs 0.94, N=45 vs N=6, p=0.007). Additionally, a trend for a higher median PI was seen in chemotherapy-treated patients compared with controls (2.43 vs 3.03, N=41 vs N=6, p=0.07). Median TAMax was lower after chemotherapy (12.65 cm/s vs 9.25 cm/s, N=42 vs N=6, p=0.04). PSV did not significantly differ between the 2 groups (36.4 cm/s vs 28.72 cm/s , N=45 vs N=6, p=0.2). Of the 6 patients who were evaluated post-chemotherapy, 1 had breast cancer, 3 had a sarcoma and 2 had a lymphoma; 1 patient was in proliferative phase, 3 patients were in secretory phase, while 2 patients were in a post-chemotherapy phase of amenorrhea.

Due to the small sample of patients included, these interesting results are to be considered merely hypothesis-generating and need to be confirmed by further analyses with due correction for confounding factors.

6. Discussion

During this PhD project, several fertility preservation strategies in female cancer patients have been investigated.

In the pre-clinical setting, we focused our attention on the supposed gonad protective role of GnRH-a concurrent administration during chemotherapy. Both pre-clinical and clinical trials on the topic led to unclear conclusions. We evaluated GnRh-a effects *in vivo* using a platform of high-resolution ultrasound imaging with contrast agents, previously validated by our group (Venturini et al., 2018). The evaluation of the mechanisms of action of GnRH-a in mice is essential for the correct interpretation of data of fertility preservation experiments using murine models.

Mice and rats have been the most used pre-clinical models in this research field, due to the large availability and ease of breeding. However, evidence on the inhibitory effect of GnRH-a on murine pituitary axis is sparse and conflicting. Some authors (Horicks et al., 2015) reported no changes in hormonal levels after GnRH-a administration, while others (Horvath et al., 2004) showed a decrease in estradiol levels, indicating at least a partial inhibition.

In our experiments, serum levels of FSH in Decapeptyl-treated mice were significantly lower at day 12 after administration, and similarly reduced levels could be detected up to day 20 after administration. However, the results of our US experiments demonstrated that GnRH-a Decapeptyl failed to inhibit folliculogenesis in mice, confirming the results reported by Horicks et al. In vivo, not only GnRH-a did not block folliculogenesis, but, paradoxically, it increased the dominant follicle diameter, an effect that was not observed in the control group.

To our knowledge, the GnRH-a-induced increase of dominant follicle diameter had never been described previously. Two different hypotheses could justify this paradoxical effect:

1. a transient flare-up of serum gonadotropin levels leading to increased follicular development. However, given the short cycle length of mice, it is highly unlikely to observe a flare-up effect after 14 days , as confirmed by Ozcelik et al

(Ozcelik et al., 2010). Possible detrimental consequences on the ovary of the GnRH-induced flare-up effect during chemotherapy have been postulated; however, in an experimental study, addition of an antagonist to inhibit flare-up did not confer a better protection against gonad toxicity (Knudtson et al., 2017).

2. a direct action of GnRH-a on the ovaries by the activation of a GnRH-a receptor expressed by granulosa cells of growing follicles. Our immunohistochemical assays using GnRHR antibodies confirmed the presence of such receptors within murine ovarian tissue. Some authors demonstrated that GnRHR is expressed by preovulatory follicles in rats (Choi et al., 2010), possibly justifying this effect.

One limitation of our study is the lack of the evaluation of estrous cycles in treated mice and controls. In any case, it is known that GnRHa can be administered at any phase of the cycle to suppress the gonadal axis. The evaluation of the estrous cycle of mice would not be particularly relevant in the present study where the effect of GnRHa was supposed to be assessed for many days after a complete mouse cycle.

Interestingly, the CEUS analysis of the average percentage change in ovarian perfusion pre-to-post treatment showed significant differences between treated mice and controls. While in the control group perfusion parameters varied consistently, in the treated group perfusion was more homogeneous with a trend toward lower values for most parameters (PE, WiR, WiPi and AUC). IF vascular staining using CD31 also revealed a reduction of blood vessels in the ovaries of treated mice versus controls.

In the second part of the experiment, a dose of 75 mg/Kg cyclophosphamide was administered to mice with or without concurrent GnRHa. At this dose, cyclophosphamide is known to destroy approximately 50% of primordial follicles (Meirow et al., 1999).

As expected, GnRHa concurrent administration did not inhibit folliculogenesis, which appeared even more pronounced in mice receiving cyclophosphamide. Among the effects of cyclophosphamide, we observed an increase of perfusion that could be reverted by concurrent GnRH administration.

At the end of this experiment, we could deduce the following observations:

1. Cyclophosphamide might increase folliculogenesis; in fact, being an alkylating agent, it is one of the most gonadotoxic drugs being involved in “burnout” theory experiments in mice (and indirectly observed in human ovarian tissue by our group

(Cioffi, Cervini, et al., 2022). Also in the rat model, cyclophosphamide showed a stimulatory effect on the ovary with an increased development of medium and large follicles, regardless of hormonal milieu (Letterie, 2004).

2. Probably, simultaneous administration of decapeptyl does not reduce the follicular burnout phenomenon because folliculogenesis is not inhibited (at least in mice).

3. Possibly, decapeptyl could be protective by reducing ovarian perfusion during chemotherapy i.e. the amount of drug being delivered to ovarian tissue. A restriction of ovarian blood flow has been previously described by Kitajima et al., 2006, as a reduction of vascular density and permeability.

Another set of mice was treated with an intraperitoneal injection of doxorubicin 2 mg/kg. Administration of doxorubicin, at least at the doses used in our experiment, did not enhance ovarian perfusion. The stimulatory effect on folliculogenesis that we observed for cyclophosphamide was not as pronounced in doxorubicin-treated mice. This result confirmed the fact that doxorubicin damages follicles indirectly, perhaps by a depletion of granulosa cells (Hasky et al., 2015). However, we noticed an increase of mTT after administration of doxorubicin, indicative of an obstructed flow of blood through the vessels, which is in fact coherent with a vascular damage. It is possible that the timepoints and drug doses decided for this study were ideal to observe changes in perfusion with cyclophosphamide administration, but not for the study of doxorubicin-induced damage.

An early impairment of blood flow was observed in mice treated with doxorubicin 8 mg/kg, starting from 3 hours after administration (Bar-Joseph et al., 2011). The microvascular damage was confirmed by histologic analysis (Soleimani et al., 2011).

In conclusion, GnRH-a decapeptyl showed some mechanisms of action that could be protective on ovarian function, especially related to perfusion, but, depending on chemotherapy class and dose, these effects vary significantly. This could partially explain the conflicting results observed in clinical trials.

Our prospective study of human ovarian cortex biopsies confirmed that chemotherapy increases the number of atretic follicles within the ovary, as expected, but it also showed an increase of primary follicles following treatment. We interpreted this as an indirect

proof of the ability of chemotherapy to disrupt follicular homeostasis by a massive over-recruitment (burn-out).

Thanks to the observation that both primordial follicles and total number of follicles were not significantly reduced in patients who started chemotherapy compared with those still waiting to receive it, we could infer the appropriateness of performing OTC even after 1 or 2 courses of chemotherapy for those patients not eligible at diagnosis, due to urgency to start treatment or clinical contraindications to laparoscopy. This also allows to postpone the decision to cryopreserve ovarian tissue in situations where the first-line treatment is not gonadotoxic, but potential shifts of treatment for resistance certainly will be.

Improving our knowledge on the potential gonadotoxicity of a treatment schedule and on patient individual factors that could influence it is extremely important. It is essential for adequate patient counselling, and it is also the base of knowledge to tailor fertility preservation strategies to maximize results and minimize risks and costs for the patient.

For this reason, we retrospectively analyzed our cohort of patients receiving counselling for fertility preservation over 10 years, which accounted for 348 patients, and collected follow-up data on menstrual activity and fertility. This allowed the development of a powerful tool to predict incidence of POF based on the presence of patient-related and treatment-related prognostic factors. We also learned how the absolute POF risk of each scheme must always be weighed on patient's age to provide precise counselling on future fertility.

In parallel, we decided to conduct separate analyses stratifying patients according to their disease, to identify the specific characteristics of each cancer diagnosis. In fact, patients with different cancers will display peculiar features and needs that should be specifically addressed by Oncofertility specialists.

First, we directed our attention toward breast cancer patients, that, due to the high incidence of disease, represent the majority of patients seeking advice on fertility and resorting to oocyte cryopreservation.

We analyzed our data to understand whether patients suffering with more advanced and aggressive cancers could have an impaired response to COS, by comparing their egg

retrieval outcomes with those of patients suffering with low-stage and low-grade breast cancer.

We found that being diagnosed with aggressive breast cancer can have an impact on basal fertility, since in our cohort of high-grade breast cancer patients the age-adjusted AFC was lower compared with low-grade disease. This also resulted in the need for higher doses of gonadotropin during COS. We did not demonstrate, however, significantly worse oocyte retrieval outcomes, which is ultimately reassuring for patients requesting this fertility preservation procedure.

Evidence on reduced basal fertility in patients with a lymphoma diagnosis can be found in the literature (Lekovich et al., 2016), while there are no data on the performance of COS in patients being diagnosed with a sarcoma.

We sought to evaluate the performance of patients suffering with a sarcoma at COS by comparing their retrieval outcomes with those of a control population. It is very hard to find healthy controls in the setting of ART, since most patients resorting to COS show different degrees of impaired fertility. The most suitable control population for this study at our center was the category of women undergoing COS for a severe male factor infertility, with no other known fertility-reducing condition. Despite the careful selection of controls and the matching by age, we cannot rule out an intrinsic bias, as these patients are not fully representative of a fertile, control population. This would, however, constitute a problem only in case we observed a lack of difference in retrieval outcomes between sarcomas and controls. Instead, the results of the analysis on our population of young patients with a sarcoma showed a trend towards better egg retrieval outcomes in the oncological cohort than in their age-matched controls. Another limit of our analysis is the less precise matching of patients aged less than 32 years, due to a generally older control population. However, this should not impact the general results of the analysis, as in terms of response to COS, age differences are less pronounced in women under 30 years when ovarian reserve is bigger.

The better performance of patients with sarcomas becomes more evident when we consider the different stimulation protocols used in the 2 groups of patients, with lower doses of gonadotropins being used in sarcomas, where risks of complications were kept

at minimum to avoid delays in cancer treatment initiation. Patients with sarcomas had a significantly lower FSH/oocyte ratio.

We can conclude that patients with a sarcoma diagnosis can expect good egg retrieval outcomes after COS, even with a random start protocol and use of sub-maximal doses of gonadotropins.

Finally, we sought to identify ultrasound uterine changes after chemotherapy in patients coming to our Clinic for counselling on fertility preservation. There are no data on uterine gonadotoxic damage, although many indirect proofs of the detrimental effects of chemotherapy on the uterus can be found (Griffiths et al., 2020)

In the literature, increasing interest is gathering around the role of increased resistance to uterine blood flow and impaired fertility. There have been some studies showing how women with unexplained infertility had higher uterine artery RI and PI compared with fertile controls (El-Mazny et al., 2013).

Our preliminary results from the first 6 ultrasound evaluations pre-to-post chemotherapy raised the interesting hypothesis that chemotherapy might increase uterine artery resistance. To our knowledge, this is the first study investigating this aspect in patients undergoing chemotherapy, therefore the prospective evaluations at our Center are currently ongoing.

6.1 Conclusions

At the present time, ensuring the best outcomes to women desiring to preserve their fertility potential means that several modalities of fertility preservation should be combined. Optimizing the opportunity for future fertility can happen by a combination of oocyte cryopreservation, OTC, and GnRH-a concurrent administration. Strategies need to be tailored to the individual profile and protection could be different depending on each drug used. Research should focus not only on the gonads, but also on the uterus, so that protective strategies could be developed on this front.

At the current state of knowledge, GnRH may be offered in addition, and not instead of other strategies.

7. Materials and methods

7.1 Murine experiments

The Animal Care and Use Committees of San Raffaele Scientific Institute, Milan, approved the murine experiments described below. Mice were housed in the air-conditioned, pathogen-free, light-controlled animal facilities of the Experimental Imaging Center of San Raffaele Scientific Institute, Milan, in accordance with EU Directive 2010/63/EU.

In the first set of experiments, 14-weeks old BALB/c mice (Charles River) were treated with a subcutaneous injection of a GnRH-a, 100 ul of Decapeptyl SR (Ipsen, Milan, Italy) equivalent to 4.45 mg/kg (n=7, experimental group) or of 100 ul of saline (0.9% NaCl) sterile solution (n=7, control group).

The two groups were compared, using US and CEUS, to evaluate ovarian morphology and vascularization at two different time-points (day 0 and day 14 from treatment). To confirm pituitary downregulation, serum protein levels of FSH were measured in Decapeptyl-treated mice and controls at day 0, day 7, day 12 and day 20. At the end of the experiments, mice were euthanized, and their ovaries were collected for immune assays.

In the second set of experiments, 12 14-weeks old BALB/c mice (Charles River) were treated simultaneously with an intraperitoneal injection of cyclophosphamide 75 mg/kg and 12 mice were treated simultaneously with an intraperitoneal injection of doxorubicin 2 mg/kg. A subcutaneous injection of a GnRH-a, 100 ul of Decapeptyl SR (Ipsen, Milan, Italy) equivalent to 4.45 mg/kg (n=6, experimental group) or of 100 ul of saline (0.9% NaCl) sterile solution (n=6, control group) was administered at day -14 and repeated at day +14 from chemotherapy. At day +30, mice were euthanized, and their ovaries were collected for histopathological examination.

The two groups were compared, using US and CEUS, to evaluate ovarian morphology and vascularization at 3 different time-points (day -15, day -1 and day +29 from

chemotherapeutic treatment). Only US was used to assess ovarian morphology and folliculogenesis at day +14, before repeating GnRH-a administration. To confirm pituitary downregulation, serum protein levels of FSH were measured in Decapeptyl-treated mice and controls at day -14, day 0, and day +30 from chemotherapeutic treatment.

Animal preparation, US, CEUS examinations and image analyses were carried out as described previously by our group (Venturini et al., 2018).

7.1.1 Hormone levels assessment

Blood samples were collected from the retro-orbital sinus. The serum was separated by centrifugation at 4000 g for 10 minutes at 4°C and stored at -80°C until further analysis. Protein levels of FSH were measured in serum samples by ELISA assay according to the manufacturer's instructions (MyBioSource, San Diego, California, USA).

7.1.2 Immunofluorescence and immunohistochemical analyses on murine ovaries

Ovaries were fixed in paraformaldehyde 4% overnight, incubated in a scale of sucrose (10% for 1 hour, 20% for 1 hour, 30% overnight), then rinsed with phosphate-buffered saline. Finally, ovarian tissue samples were embedded in OCT (TissueTek) and frozen in liquid nitrogen. Sections were counterstained with hematoxylin and eosin. Ovarian tissue samples were stained in immunofluorescence (IF) with rat anti mouse CD31 LS-C96348 LSBio) and visualized with a Goat anti-Rat IgG Secondary Antibody, Alexa Fluor 594 and Hoechst 33342 as counterstaining.

The sections were observed on a Nikon Eclipse 55i microscope (Nikon, Tokyo, Japan) and Images were captured by Nikon Digital Sight DS-5M 5MP Microscope Camera using Lucia G software (Laboratory Imaging, Prague, CZ). Parallel slides in which primary Ab had been omitted were identically processed and used as negative controls.

For immunohistochemistry, sections were incubated with antibodies against GnRHR (GRX-8) /sc-69847 and GnRHR (C18)/sc-8681 (Biotechnology, Inc., Santa Cruz, CA,

USA) using M.O.M. (Mouse on Mouse) detection kit protocol. The negative control sections were treated identically to all other slides, but the primary antibodies were exchanged with their corresponding primary isotype mouse IgG antibodies (Santa-Cruz Biotechnology).

7.1.3 Statistical analysis

Statistical analysis was performed by Student's *t* test with two-tailed distribution and two-sample unequal variance for US and CEUS data, and by Mann-Whitney and Kruskal-Wallis tests for hormone levels assessment. *F* test was used to compare variances. Bonferroni test was used to correct for multiple comparisons (GraphPad Prism 5.04 software). Data are presented as mean ± standard deviation *(*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ and ****, $P < 0.0001$).*

7.2 Histological analysis on human ovarian tissue

Tissue harvesting and processing and methods of follicular count have been described in our recently published paper (Cioffi, Cervini, et al., 2022).

7.3 Controlled ovarian stimulation

COS protocols used at our Fertility Preservation Unit have been reported in detail in our publication (Cioffi et al., 2021).

7.4 Other statistical analyses

Statistical analyses were carried out using the IBM SPSS Statistics software, version 27 for Mac (SPSS, Chicago, IL). Shapiro-Wilk test was used to verify normal distribution of the data. When data was normally distributed, t-test and one-way analysis of variance (ANOVA) were used for continuous variables. When data distribution was not normal, Mann-Whitney test and Kruskal-Wallis test were used to compare continuous variables. Fisher's exact test/chi-square test were used to compare categorical variables. Univariate binary logistic regression was used to correct all the analyses by age.

For the analysis on the population of sarcomas, the database of cases and controls was created using R version 4.1.2, performing a match by group without replacement (*RStudio Team*). Subsequently, univariate comparisons were performed using Mann-Whitney test, and multivariate comparisons using linear regression in SPSS v.27 for Mac.

For the definition of the predictive model, a multivariate logistic regression analysis using forward conditional mode was performed (enter variable p-value: 0.1; remove variable p-value: 0.2). Propensity scores were deducted. A regression receiver operating characteristic curve (ROC) was constructed, with measurement of area under the curve (AUC) and corresponding 95% confidence intervals. All calculated p-values were two-sided and p-values ≤ 0.05 were considered statistically significant.

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