Oncofertility Procedures Department of Gynecological Endocrinology and Human Reproduction, University Hospital of Split

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Diploma thesis

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This thesis is made in cooperation with the Department of Obstetrics and Gynecology, University Hospital of Split, led by Assist. Prof. Jelena Marušić and submitted for assessment in the academic year of 2017/2018. of medical school. First and foremost, I would like to express my special appreciation and thanks to my advisor Assist. Prof. Jelena Marušić for your support and guidance. My gratitude also goes to the Department for giving me the opportunity to collect data even though my research sometimes lasted beyond working hours. Next, I want to thank my two roommates for always being there for me, helping me and motivating me to carry on. Finally, I would like to thank my family: my parents and my sister. They give me nothing but support, emotionally and spiritually, and encourage me to do my best every day. My love and gratitude for you can hardly be expressed in words.

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1. INTRODUCTION

1.1. Overview oncofertility

Today, patients can look forward to life after malignant diseases such as cancer, yet many may face the possibility of infertility as a result of the disease itself or its lifesaving treatments (1). Oncofertility has emerged as a new branch of medicine, with the task of developing a safe and effective method of preserving fertility in men and women. The name was first used by Teresa K. Woodruff in 2006 and since then oncofertility developed as a new discipline of gynecology and obstetrics (2). The need for further investment in this branch has two major reasons. First concern is the increasing incidence of malignant diseases in children and young adults. Secondly, advances in cancer diagnosis and the introduction of new cancer treatments have dramatically improved the chance of survival, allowing patients and practitioners to think beyond the cure to future quality of life. Unfortunately, to treat these malignant diseases, aggressive therapy is necessary, which causes a marked reduction in reproductive potential.

In 2006, it was estimated that around 750 000 young women only in the USA had to undergo cancer treatment during their childbearing age. Among these female cancer survivors who were under the age of 40 at diagnosis, the chance of getting pregnant was 20% lower in those diagnosed as children, and 50% lower in those diagnosed as young adults, compared to female siblings without cancer. Those numbers seen in a global dimension, represent a tremendous unmet need regarding the level of infertility and its extent on survivors' quality of life (3).

Also, the number of young patients whose reproductive futures may be affected by cancer treatment or the cancer itself is not small. In 2016 approximately 9 million people were diagnosed with malignant neoplasms worldwide, and around 1,1 million diagnoses were made in patients younger than 45 years of age (4), a time when many may be thinking about or actively building their families. Especially these patients need reproductive consult at the time of diagnosis to address options for preserving fertility before cancer treatment begins (3).
Most impressive survival statistics can be found in children diagnosed with cancer. Childhood cancer patients (age 0–14 years) have an average 85% chance of survival. For this reason, addressing the late effects associated with cancer treatment in these prepubertal patients, such as reproductive and endocrine issues, has also taken an important part in today's medicine (3).

The office for National Statistics in Great Britain found out that leukemia, non-Hodgkin's lymphoma and brain were the most common cancers registered in young boys (0 to

14 years old). In young girls, the kidney was the most common site. These cancers accounted for over half of the 1,359 cancer cases registered in children.
For males aged 15 to 49, testicular, skin melanoma, and bowel cancer were the 3 most common cancer registrations. For females the same age the most prevalent cancer sites were breast, skin melanoma and cervical cancer.

In older patients, being 50+ respectively, prostate, breast, lung, and colorectal cancer were the most common cancer cases registered (5).

1.2. Gonad toxicity

It was shown that chemotherapy and radiotherapy have adverse effects on the organs of men and women as they can lead to complete dysfunction of the testis or ovaries respectively, resulting in infertility. Infertility is defined as the inability to conceive within a period of 12 months of unprotected sex. Gonad toxicity can also cause the advent of premature menopause (before the age of 40) due to premature ovarian failure (POF) and prevent puberty from proceeding normally (3).

1.3. Radiotherapy

Ionizing radiation causes direct damage to deoxyribonucleic acid (DNA) leading to cell damage and death. Radiosensitivity is highest in cells which are highly mitotic or undifferentiated. For this reason, the gonads are considered highly radiosensitive and are therefore very susceptible for radiotherapy. At the time of birth, the ovaries contain a finite number of follicles, which accounts for about 2 million. That number continues to decrease until menopause. Destroying all follicles will cause irreversible infertility. The schedule of the delivered irradiation (total dose, number of fractions, and duration) is an important determinant of the radiobiological effect on the tissues involved and varies among different tissues and organs. Irradiation to the central nervous system may affect the timing of the onset of puberty, result in hyperprolactinemia, or cause gonadotropin deficiency if the hypothalamic-pituitary axis is involved in the radiation field (6). Direct irradiation to the testis will, in lower doses, affect the germinal epithelium. Doses of irradiation greater than 0.35 Gy will lead to a temporary azoospermia. The time until normal spermatogenesis is reinstalled, increases with larger doses. However, with doses more than 2 Gy the absence of sperm may be permanent. At very high radiation doses (> 15 Gy), Leydig cell function will also be affected. In addition to radiation dose, the damage potential of the testis is dependent on the age at irradiation and the

pubertal status of the male. In females, the vulnerability of the ovary to irradiation varies with age as well as dose, and separation of ovarian dysfunction into hormonal and fertility effects is not clear-cut. An ovarian dose of 4 Gy may cause a 30% incidence of sterility in young women, but 100% sterility in women over 40 years of age. Pelvic irradiation may also have a significant effect on the uterus, with restricted growth in the prepubertal girl, and failure of uterine expansion during pregnancy leading to miscarriages and premature labor (7).

1.4. Chemotherapy

Chemotherapy damages the ovaries in three ways. We can distinguish between direct damage of the ovary, ovarian damage as a result of vascular damage and damage at the cell level (oxidative stress) (6). Direct damage causes impairment of follicular maturation or direct toxicity on quiescent primordial follicles or the combination of two, resulting in an overall reduction of oocytes and a temporary loss of menstruation, the so-called *burn-out effect*. The clinical impact of chemotherapeutic drugs on the ovary is variable, ranging from no effect to complete ovarian atrophy. However, the degree of damage is usually dependent upon the type of the chemotherapeutic agent used, dose given, age of the patient and the baseline ovarian reserve. The prepubertal ovary is less susceptible to the sequelae of chemotherapeutic agents while older women, having a lower ovarian reserve, are more susceptible to premature ovarian failure (POF) (7).

In women who are 20 years old, it took about 20,400 mg cyclophosphamide for the occurrence of amenorrhea, in women 30 years old, 9300 mg, and for women of 40 years only 5200 mg of cyclophosphamide (8). The groups of chemotherapeutic agents can differ in the mode of action, as demonstrated in Figure 1, and consequently have adverse effects on the reproductive system. Alkylating agents have an extremely damaging effect and are responsible for the highest age-adjusted odds ratio of ovarian failure rates.

Platinum-based compounds such as cisplatin cause DNA damage. They carry a medium risk of amenorrhea. Anthracycline antibiotics such as doxorubicin (DXR) induce oxidative stress. The amenorrhea and fertility risk are medium to low with this group of drugs (9).

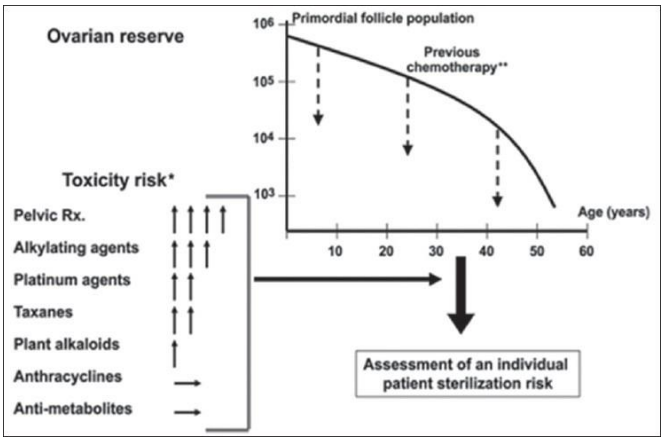

Figure 1. Cytotoxic drugs and their action on the ovary

Reprinted from: Mahajan N. Fertility preservation in female cancer patients: An overview. Journal of Human Reproductive Sciences. 2015;8(1):3-13.

Fibrosis of blood vessels due to toxic agents add to the ovarian damage. Ben-Aharon et al. followed 20 women during and after chemotherapy with a focus on vascular damage. They found out that ovarian toxicity manifested by decreased ovarian blood flow accompanied by a reduction in ovarian size and diminished post-treatment anti-Müllerian hormone (AMH) levels (10).

The endogenous free oxygen radicals have proved to be crucial factors in apoptosis of antral follicles in response to ionizing radiation and toxins, reducing fertility in women who received chemotherapy (11).

The normalization of the menstrual cycle after the treatment ensures normal reproductive capacity. It is important to note that the patients who received gonadotoxic treatments usually enter menopause earlier than healthy women (12). There is a risk of post chemotherapy loss of ovarian function several years after the treatment of malignant disease (10).

Chemotherapeutic agents also have detrimental effects on spermatogenesis, causing temporary, long-term, or permanent gonadal toxicity in men. The relatively long time span of loss of functional sperm production, is based on the fact that the rapidly dividing differentiating spermatogonia are much more sensitive to killing than later stage germ cells, and that the kinetics of spermatogenesis is fixed and unchanged after cytotoxic treatments. Thus, the surviving later stage germ cells progress along their differentiation pathway but are not replaced by new cells that would have been derived from the differentiating spermatogonia that have been destructed. Hence there is a progressive loss of the more mature differentiating cells in a process called maturation depletion.

Alkylating drugs are the most potent at producing long-term azoospermia. In male patients, who are being treated for testicular cancer a common choice of chemotherapy is either based on cisplatin or carboplatin. Lampe et al. analyzed data concerning 170 patients with testicular germ cell cancers who underwent treatment with either cisplatin- or carboplatin-based chemotherapy. Forty of these men (24%) were azoospermic before the treatment, and a further 41 (24%) was being found to be oligospermic. A median of 30 months after the completion of chemotherapy, only 64% of those patients who had a normal sperm count before therapy remained normospermic, while 54 (32%) of the total cohort were azoospermic and 43 (25%) were oligospermic (13). The probability of recovery to normospermia appeared to be higher for those men with a normal pretreatment sperm count, in those who received carboplatin- rather than cisplatin-based therapy, and in those treated with fewer than five cycles of chemotherapy. Recovery of the patients lasted for more than 2 years, with the calculated chance of spermatogenesis at 2 years being 48% and at 5 years 80% (14).

1.5. Process of cryopreservation

Cryopreservation (CP) is the freezing of gametes, embryos and testicular tissues to maintain their viability over time. The first step is cooling the temperature of 37 ° C to a temperature of -196 ° C. Second step is to re-thaw the sample to a temperature of 37 ° C after a certain period, meaning when the patient wishes to use the sample (8). The biggest obstacle during this process is to remove the water successfully from the cells without causing their death. Freezing water increases its volume and can lead to rupture of the cell membranes and damaged organelles within the cell. Therefore, before freezing the water needs to be replaced by a cryoprotectant medium. There are penetrating and non-penetrating cryoprotectants. Penetrating cryoprotectants cause membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and increased survival to cryopreservation. Additionally, penetrating cryoprotectants are solvents that dissolve sugars and salts in the cryopreservation medium. A non-penetrating cryoprotectant does not cross plasma membrane and only acts extracellularly. Therefore, non-penetrating cryoprotectant may alter the plasma membrane, or act as a solute, lowering the freezing temperature of the medium and decreasing the extracellular ice formation (15). There are two methods to do cryopreservation, slow freezing and vitrification. Slow freezing is the conventional method in which the temperature drops slowly, by 0.3 ° C per minute to the desired temperature of -32 ° C to ensure the tissue is dehydrated before

intracellular ice formation occurs. After that, the sample is transferred to the liquid nitrogen temperature of -196 ° C. Whereas in conventional cryopreservation the concentration of the cryoprotectant is low and the cooling rate is very slow to avoid ice crystallization, vitrification is an ultra-rapid cooling technique that requires a high concentration of cryoprotectant (16).

Figure 2 compares the survival rates of oocytes in 5 different studies, using slow freezing or vitrification:

Egg Freezing Survival Rates	Slow Freeze	Vitrification
Cobo et al. 2008	-	97%
Rienzi et al. 2010	-	97%
Cao et al. 2009	62%	92%
Smith et al. 2010	65%	75%
Cobo et al. 2010	-	93%

Figure 2. A summary of randomized controlled trials reporting the egg survival rate of slow freezing and vitrification

Reprinted from: http://www.smartfertilitychoices.com/your-ultimate-guide-to-egg-freezing/

1.6. Oncofertility procedures

1.6.1. Cryopreservation of embryos and oocytes

1.6.1.1. Controlled ovarian stimulation

Cryopreservation of embryos and cryopreservation of oocytes are both methods of preserving fertility in women. It is considered as the golden standard for patients threatened with the loss of ovarian function due to gonadotoxic therapy (17). The common step at the beginning of both methods is controlled ovarian stimulation (COS). Before treatment starts, the patient should undergo thorough screening and check-up. In addition to determining the type of tumor and its stage, it is of great significance to estimate the time remaining until commencement of cancer therapy. Also, the patient's ovarian reserve should be calculated to identify whether the method is fitting for that specific patient. It is usually determined by the number of antral follicles, AMH levels and follicle stimulating hormone (FSH) (18). Nowadays, we use AMH levels and the antral follicular count to predict the success of in vitro fertilization (IVF). AMH markers are considered more accurate because its levels do not change during the cycle but can serve as a marker at any time (19). Controlled ovarian stimulation is a method in which exogenous hormones are administered to achieve synchronized growth and development of several follicles at once. This ensures a greater number of released mature

oocyte per cycle. Controlled ovarian stimulation comprises three main elements: Exogenous gonadotropins to stimulate multi-follicular development, cotreatment with either gonadotropin-releasing hormone (GnRH) agonist or antagonists, to suppress pituitary function and prevent premature ovulation by a luteinizing hormone (LH) surge, and finally triggering oocyte maturation 36 to 38 hours prior to oocyte retrieval.

GnRH agonists or antagonists have been used in a number of different protocols. In the so-called 'long protocol', the GnRH agonist is started at least two weeks before stimulation and continued up until oocyte maturation is achieved. Alternatively, a 'short protocol' is used in which the GnRH agonist is commenced simultaneously with stimulation and continued up until the day of oocyte maturation trigger. Yet another option is the use of GnRH antagonists. This involves a shorter duration of use compared with the agonist 'long protocol' and is started a few days after initiation of stimulation, continuing up until administration of the drug triggering oocyte maturation. The trigger is used at the end of the stimulation phase of an IVF cycle and mimics the natural endogenous LH surge in order to initiate the process of ovulation. Two drugs are currently used: human chorionic gonadotropin (hCG), which is the most common drug, or GnRH agonist in an antagonist protocol (17).

In today's protocols exogenous gonadotropins are administered daily for 9-14 days, starting in the early follicular phase. As already mentioned before, the second step would be to prevent the premature ovulation by administering GnRH agonists during the luteal phase. In addition to GnRH agonists, the use of GnRH antagonists to prevent ovulation has been evaluated. Antagonists are administered when the leading follicle diameter is 12-14 mm, which is usually by the sixth day of stimulation. The use of a GnRH antagonist shortens the process of ovarian stimulation markedly by 14 days which is a great advantage compared to GnRH agonists (20). The different proceedings are illustrated in Figure 3:

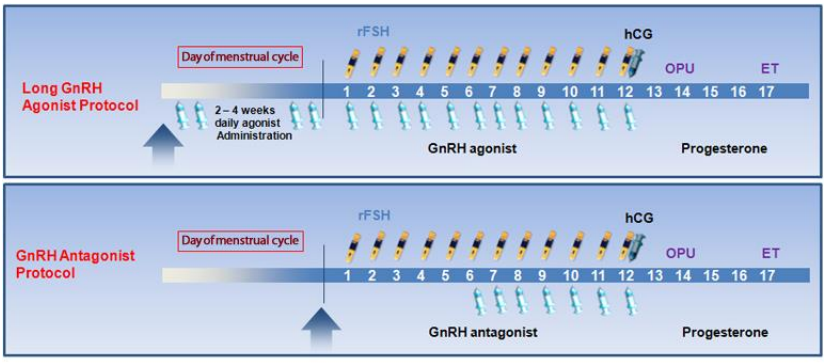

Figure 3. Suppression of spontaneous ovulation

Reprinted from: http://www.motherhoodconsultancy.com/controlled-ovarian-hyperstimulation/

In the GnRH agonist protocol, hCG is applied intramuscularly once the leading follicle has reached an appropriate size. Aspiration of oocytes is then performed 36 to 38 hours after the time of administration of hCG (21).

In addition to the conventional protocol, a non-conventional protocol has been developed. Unlike the standard protocol, in which patients must be in early follicular phase to start stimulation, patients can also begin in late follicular or luteal phase of the cycle. This is also called random-start COS and has been reported as an alternative method for cancer patients, with the benefit of a shortened interval from presentation to retrieval and thus to cancer treatment. The conventional approach requires approximately 2 weeks of ovulation induction from the beginning of the menstrual cycle. This process could entail a delay by up to 6 weeks from starting cancer treatment, depending on the phase of the menstrual cycle during which the patient is referred (22).

Baerwald et al. followed the ovaries of 50 healthy women in their reproductive age every day by transvaginal ultrasound over the period of one interovulatory interval (IOI). They found out that women exhibit a follicular wave phenomenon, in which follicles not only grow during the follicular phase of the menstrual cycle but show major and minor waves of growth ranging from two to three ovarian follicular waves during one interval. This means that the selection of a dominant follicle for preferential growth and development to an ostensibly ovulatory diameter can occur at more than one time during the menstrual cycle. Only the final wave of each cycle was ovulatory; all preceding waves were anovulatory (23). This opens two additional starting points for controlled ovarian stimulation in patients who might not be able to wait 2-6 weeks: the late follicular phase and/or luteal phase. The first option is to start the stimulation after a spontaneous LH surge. The second option is after ovulation, induced by administration of a GnRH agonist or hCG. In the random start-protocol, like in the conventional one, GnRH antagonists are used later in the cycle, or if the diameter of the follicles is larger than 12 mm. The task of GnRH antagonists is the prevention of a LH surge, and the application is continued until ovulation induction with GnRH agonists or hCG is started. The only exception is its use in the late follicular phase, if the diameter of follicles reached 12 mm before the spontaneous LH surge. In this case, administration of a GnRH antagonist starts immediately and continues until ovulation induction (24). Jee Hyun Kim et al. evaluated the efficacy of random-start controlled ovarian stimulation (COS) in cancer patients for emergency fertility preservation. In this retrospective comparative study they included 22 patients diagnosed with cancer and 44 infertile women undergoing conventional in vitro fertilization (IVF). In cancer patients, ovarian stimulation was started on the day of referral, irrespective of their menstrual

cycle date. The control group was selected by age matching among women undergoing conventional IVF. COS outcomes were compared between groups. The number of total and mature oocytes retrieved, and the oocyte maturity rate were higher in the random-start group than in the conventional-start group. However, ovarian stimulation took more time in the random-start group (11.4 vs. 10.3 days, $p = 0.004$). The results confirm the feasibility and effectiveness of random-start COS in cancer patients and proves that progesterone levels in the luteal phase and the presence of the corpus luteum have no negative effects on the development of oocytes (25). It is therefore of utmost importance to familiarize cancer patients with the possibility of preserving fertility, about its process, and to point out that it does not significantly delay therapy.

In oncology, hormone-sensitive tumors make up an exceptional group. The most common one that occurs in women is breast cancer, followed by endometrial cancer. The percentage of breast tumors that are sensitive to hormones is up to 60%. To identify those patients is exceptionally important for their further treatment, as hormones binding to these receptors enhance tumor cell proliferation. Stimulation of the ovaries increase the number of antral follicles. This leads to an increased number and proliferation of granulosa cells (8). These cells contain the enzyme aromatase, and its membrane the receptors for FSH. FSH binding to the receptors, activate the enzyme aromatase, and thus converts androstenedione to estrone and testosterone to estradiol. In the normal menstrual cycle, the peak concentration of estradiol is around 300 pg/mL, in COS, however, values of 3000 pg/mL can be reached, arising concerns that the stimulation of ovulation would further encourage tumor growth and increase mortality. Therefore, alternative and potentially safer protocols have been introduced for fertility preservation in estrogen-sensitive cancer patients, including natural-cycle IVF (without ovarian stimulation), stimulation protocols with tamoxifen alone or combined with gonadotropins, and stimulation protocols with aromatase inhibitors to reduce the estrogen production (24).

Natural-cycle IVF gives only one or two oocytes or embryos per cycle and has a high rate of cycle cancellation. Therefore, this technique can be considered as likely ineffective and is not recommended, especially when a chemotherapy treatment is pending, and the patient does not have a chance for a second cycle of IVF treatment (24).

Tamoxifen, a nonsteroidal triphenylethylene compound, has an antiestrogenic action on breast tissue. It inhibits the growth of breast tumors by competitive antagonism of estrogen at its receptor site. It is accepted as the first-line drug in hormonal prevention and treatment of estrogen receptor–positive breast cancer. Tamoxifen, besides its effect in the breast, also has an

antagonist action in the estrogen receptors in the central nervous system. The selective antagonist action of tamoxifen intervenes with the negative feedback of estrogen on the hypothalamic-pituitary axis, resulting in an increase in GnRH secretion from the hypothalamus and a subsequent release of FSH. Tamoxifen can be used for COS alone starting on day 2–5 of the menstrual cycle in doses of 20–60 mg/d, or in combination with gonadotropins. Even though peak estradiol levels in ovarian stimulation with tamoxifen are not altered, owing to its antiestrogenic effect on breast tissue, it should preferably used in estrogen receptor–positive breast cancer patients. In a study of Oktay et al., ovarian stimulation with the use of tamoxifen for fertility preservation in cancer patients was shown to increase the mature oocyte and embryo yield compared with natural-cycle IVF (1.6 vs. 0.7 and 1.6 vs. 0.6, respectively) and reduce cycle cancellations. Combined protocol with tamoxifen and gonadotropins further increased the number of cryopreserved oocytes and embryos (5.1 vs. 1.5 and 3.8 vs. 1.3, respectively) (26).

Stimulation protocols with aromatase inhibitors, such as letrozole, markedly suppress plasma estrogen levels by competitively inhibiting the activity of the aromatase enzyme. Aromatase is a cytochrome P450 enzyme complex that catalyzes the conversion of androstenedione and testosterone to their respective estrogenic products estrone and estradiol (27). Aromatase inhibitors significantly reduce the risk of recurrence in postmenopausal women with hormone receptor–positive breast cancer owing to profound estrogen deprivation (28). Centrally, aromatase inhibitors rid the hypothalamic-pituitary axis of the estrogenic negative feedback, increase the secretion of FSH by the pituitary gland, stimulate follicle growth, and therefore make it suitable for ovulation induction (29). In patients with estrogen-sensitive cancers, the main advantage of adding daily letrozole to gonadotropins in ovarian stimulation protocols is to decrease serum estradiol levels to be closer to that observed in natural cycles without affecting oocyte or embryo yield (30,31). Stimulation protocols using letrozole alongside with gonadotropins are currently preferred over tamoxifen protocols as treatment with letrozole results in a higher number of oocytes obtained and fertilized when compared to tamoxifen protocols (26). A study of Oktay et al. compares the efficacy of the letrozole plus gonadotropin protocol in breast cancer patients and the standard IVF protocol in age-matched noncancer patients with tubal-factor infertility. The breast cancer patients started to receive letrozole (5 mg/d) on menstrual cycle day 2 or 3, and FSH (150–300 IU/d) was added 2 days later. All medications were discontinued on the day of hCG trigger, and letrozole was reinitiated after oocyte retrieval and continued until estradiol levels fell to <50 pg/mL (31). This protocol resulted in similar number of total oocytes retrieved and length of ovarian stimulation compared with standard IVF protocol (31). Peak estradiol levels were shown to be significantly lower

in the breast cancer patients receiving letrozole plus gonadotropin compared with the standard IVF group (483 ± 278.9 pg/mL vs. 1,464.6 ± 644.9 pg/mL). Other studies assessed the effect of letrozole on oocyte maturity and competence. They demonstrated that the addition of letrozole did not change numbers of mature oocytes retrieved and fertilization rates (31,32). Azim et al. further found out that the short-term follow-up of breast cancer patients, who had undergone ovarian stimulation with letrozole plus gonadotropins for fertility preservation, has not revealed a raise in the risk of breast cancer recurrence (33). In addition, COS with letrozole in combination with gonadotropins has been safely used for embryo cryopreservation in endometrial cancer patients (34). Discontinuation of letrozole can either be at menses or with initiation of chemotherapy. In contrast, anastrozole, another third-generation aromatase inhibitor, failed to adequately suppress estradiol levels during COS, despite gradually increasing the dose to a maximum, and therefore its use is not recommended in fertility preservation cycles (35).

In summary, COS with letrozole plus gonadotropins in patients with estrogen-sensitive cancers undergoing fertility preservation is safe, well-tolerated, and yields similar number of oocytes and embryos compared to standard protocols while minimizing the risk of high estrogen exposure and not increasing the risk of recurrence of cancer in the short term.

Carriers of the BRCA gene make up another group of special cancer patients in need of an adjusted therapy. BRCA genes play an essential role in double-strand DNA break repair, and their mutations are associated with an increased risk of breast and ovarian cancers. Therefore, in patients with BRCA mutations, oocytes may be more prone to DNA damage, clinically manifesting as diminished ovarian reserve or earlier menopause. In BRCA mutation–positive breast cancer patients, Oktay et al. found out that a low response to ovarian stimulation occurred more frequently than in patients without BRCA mutations (33.3% vs. 3.3%) or in breast cancer patients not tested for their BRCA status (2.9%). Interestingly, all BRCA mutation–positive patients with a low response to ovarian stimulation and requiring higher doses of gonadotropins for their stimulation had BRCA-1 mutations. A low response was not encountered in women who were positive for only BRCA-2 (36).

Ovarian hyperstimulation syndrome (OHSS) is the most serious complication of ovarian stimulation and can be associated with intravascular depletion, ascites, liver dysfunction, pulmonary edema, electrolyte imbalance, and thromboembolic events. Although OHSS is often self-limited with spontaneous resolution within a few days, severe disease can require hospitalization and intensive care. The impact can be especially profound in cancer patients because it may result in a delay or complications of imminent cancer therapy (37).

Triggering the final oocyte maturation with hCG carries the risk of inducing OHSS (38). GnRH agonist also induces this final oocyte maturation by promoting the release of endogenous gonadotropin stores from the pituitary gland, as long as its gonadotropin receptors are not down-regulated. It can therefore be used as an alternative to hCG (38), which dramatically reduces the risk of OHSS, owing to the short half-life of a GnRH agonist–induced endogenous LH surge (39). In a study of Oktay et al., comparing GnRH agonist and hCG as the trigger for oocyte maturation in fertility preservation cycles, GnRH agonist trigger resulted in at least similar numbers of mature oocytes and cryopreserved embryos compared to hCG (40). In addition, although hCG potentiates the endogenous production of estrogen during the luteal phase owing to its longer half-life, GnRH agonist–induced endogenous LH may result in lower estrogen production, which may be an advantage for patients with estrogen-sensitive cancers (38). However, the use of GnRH agonists may result in a failed induction of ovulation (41). The expected risk lies between 1.4 and 3.5% (42). This can be prevented by either higher doses of GnRH agonists or hCG can be added to induce ovulation (41).

1.6.1.2. Cryopreservation of embryos

The ability to cryopreserve, thaw, and accomplish pregnancies with supernumerary preimplantation embryos has become a valuable tool in fertility treatment. In a routine IVF practice, 60 % of stimulated IVF cycles may achieve surplus embryos suitable for cryopreservation (43). There are two methods of cryopreservation: slow-rate freezing and vitrification. Vitrification is now considered superior to the traditional slow-rate freezing. Survival rate of cleavage stage embryos was significantly higher after vitrification as compared with slow freezing. Vitrifying 1600 embryos, Kuleshova and Lopata reported a 84% survival rate with a 51% pregnancy rate (44). Desai et al performed a study of vitrification on human embryos at 6–8 cell-stages. The post-warming survival rate was 85%, the implantation rate was 19.9% and the clinical pregnancy rate was 44.2% . Also post thawing survival rate of vitrified blastocysts was significantly higher compared with that observed with slow freezing (45). Liebermann and Tucker had reported in their study 96.5%, 30.6%, and 88.6% for survival, implantation, and pregnancy rates respectively (46).

Besides, studies of Riggs et al. have demonstrated no correlation between embryo storage duration and the live birth rate (47), which is soothing for oncology patients, given they often have to delay pregnancy to complete therapy and due to concerns over cancer recurrence (48).

Cardozo et al. compared in-vitro fertilization (IVF) outcomes of cancer patients who underwent oocyte retrieval and embryo cryopreservation prior to gonadotoxic therapy to those of age and time-matched controls with tubal factor infertility. Out of sixty-three cancer patients, 21 returned for frozen embryo transfer. In the control group of 122 age-matched patients, 23 returned for frozen embryo transfer. No difference was seen between cancer patients and controls with respect to number of oocytes retrieved and number of embryos obtained. The pregnancy rate per transfer for cancer patients compared to controls was 37 vs. 43 % respectively, and the live birth rate per transfer was 30 vs. 32 % respectively (49). These results clearly show the efficacy and utility of this method for cancer patients.

Although considered the golden standard, it shows some limitations. One would be the necessity for COS and the time it takes to perform it, which some cancer patients might not have. There is also the need for a male partner, which can produce viable sperm. Finally, the storage of embryos has elicited religious and ethical issues, as well as concerns regarding cost-effectiveness, resulting from high disposal and non-usage of embryos (50).

1.6.1.3. Cryopreservation of oocytes

Mature oocyte cryopreservation is a currently available method of fertility preservation in women of reproductive age. Even though it efficacies remains low compared to embryo CP, it should be preferred when the latter is prohibited by law, avoided for ethical or religious issues and in single women refusing sperm donation (43). It also helps to overcome problems, for example when the husband is unable to produce a viable sperm sample or when spermatozoa cannot be found in the testis in case of non-obstructive azoospermia. Moreover, with the help of oocyte CP, not only women but also young female cancer patients could store their gametes before undergoing gonadotoxic therapy. In all these cases, banking mature oocytes is a reasonable fertility-preserving alternative. In the past 10 years, methods of vitrification have been refined to optimize oocyte survival after cryopreservation (51-54). Both clinical trials and observational studies have compared reproductive outcomes after IVF and intracytoplasmic sperm injection (ICSI) with cryopreserved oocytes to IVF and ICSI with fresh oocytes. Outcomes of four published randomized controlled trials demonstrated that fresh and frozen oocytes yield similar pregnancy rates in IVF cycles, supporting the use of these technologies in selected patients aged 35 years and younger (55-58). In the two studies conducted in infertile couples, implantation rates ranged between 17% and 41% and clinical pregnancy rates per transfer ranged from 36% to 65% (56, 58). These data, and data from a recent meta-analysis

(59) suggest that specific outcomes of IVF and ICSI (fertilization and pregnancy rates) are similar between fresh oocytes and vitrified oocytes. An important clinical predictor of outcomes in the observational studies of oocyte cryopreservation and IVF is the age of the oocyte when frozen or vitrified (60-63). Several studies have displayed that a more advanced age of the oocyte, when frozen or vitrified, reduces the odds of success when used for IVF or ICSI.

Oktay et al. analyzed the efficacy of oocyte cryopreservation in cancer patients, by slow-freezing and vitrification methods using a meta-analysis. They reported live birth rates per oocyte thawed of 3.4 % for slow freezing and 6.6 % for vitrification (64). These results suggest that acceptable live births after oocyte cryopreservation can occur in cancer patients. However, it is important to note that most retrospective studies on successful oocyte cryopreservation in cancer patients have been case reports. Therefore, it is possible that results that did show an unsuccessful pregnancy were excluded. Due to this, it is still difficult to predict the possibilities of having a live birth. Further long-term follow-up studies on cancer patients would more accurately determine the efficiency of oocyte cryopreservation.

Today, oocyte cryopreservation, with appropriate counseling, is recommended for patients facing infertility due to chemotherapy or other gonadotoxic therapies, and who do not wish or cannot perform embryo CP. Unfortunately, despite the increasing use of this strategy, data are still lacking about the efficacy and safety of the procedure in female cancer patients. Also the age of the oocyte when vitrified has shown to play a major role in a successful pregnancy, and this method is therefore more recommendable for younger patients.

1.6.2. Ovarian tissue freezing

Ovarian tissue cryopreservation is still an experimental, but rapidly progressing technique (64). For ovarian tissue cryopreservation, ovarian tissue is obtained laparoscopically and cut into cortical strips which contain many primordial follicles. The tissue is then cryopreserved either by slow freezing or vitrification (65). Ovarian tissue can be drawn from any female patient irrespective of their age, while mature oocytes required for embryo or oocyte cryopreservation can be harvested only from postpubertal women. Ovarian tissue cryopreservation can furthermore be conducted within a few days because it is COS-independent, while COS-dependent embryo or oocyte cryopreservation requires at least two weeks, no matter the protocol. Moreover, ovarian tissue cryopreservation comprises the possibility of being combined with embryo or oocyte cryopreservation (65). Ovarian tissue cryopreservation followed directly by COS and oocyte retrieval did not impair the number or

quality of the retrieved oocytes in cancer patients, as shown in a study of Dolman et al. (66). This combination may increase fertility preservation potential. When a patient wants to avail her cryopreserved ovarian tissues, they are thawed and transplanted. The most common place is in the pelvic cavity, either on the ovarian medulla or inside a peritoneal window (65,67,68). It has been shown that after transplantation of ovarian tissue in the pelvic cavity, ovarian endocrine activity is restored in more than 95% of cases (65, 69). As of the beginning of 2018, more than 130 live births after transplantation of ovarian tissue, cryopreserved either for oncologic or non-oncologic reasons, has been reported (69). A pregnancy rate after transplantation of ovarian tissue is not established since the number of transplantations performed worldwide is not known. However, a pregnancy rate and live birth rate are estimated, based on the results of several case series as 30–40% and 25–35%, respectively (65, 70–73). Pregnancies after ovarian tissue preservation resulted either from natural conception or from in vitro fertilization (74). The biggest concern regarding transplantation of cryopreserved ovarian tissue for oncologic reasons is the risk of reintroducing malignant cells contaminating the ovarian tissue, which is called minimal residual disease (MRD). The relative MRD risk for most types of cancer is still unknown, but the risk of MRD is considered to be high in leukemia patients (75, 76), moderate for gastrointestinal cancer, and low for breast cancer, sarcomas of the bone and connective tissue, Hodgkin's and non-Hodgkin's lymphoma (77). In addition, the methods to evaluate MRD in ovarian tissue is not established. Accordingly, ovarian tissue cryopreservation and transplantation is not recommended for leukemia patients at present (78). Ovarian tissue freezing is indicated in selected patients scheduled for treatments with a high risk of premature ovarian failure. This procedure can also be used in patients who are concerned and do not wish to undergo COS, or women with difficulties to undergo vaginal ultrasound examination. Furthermore, the technique can be proposed in patients who cannot delay the initiation of anticancer treatments (e.g. women diagnosed with an aggressive form of early breast cancer at a more advanced stage). Finally, it should be noted that, this is the only available option in prepubertal girls who are candidates for gonadotoxic treatments (79).

1.6.3. GnRH agonists

Administration of GnRH agonist has been considered a pharmacologic protection of the ovary during chemotherapy. The proposed mechanisms of action include hypogonadotropism-induced ovarian quiescence and reduction of ovarian blood flow. Although GnRH agonist may have some medical benefits such as prevention of menorrhagia induced by thrombocytopenia

following chemotherapy, there is insufficient evidence regarding the effectiveness of GnRH agonist in fertility preservation (78). Several papers with conflicting results have continued to be published (80-82), but to confirm the protective effect of GnRH agonist, it would be necessary to show that administration of GnRH agonist is effective not only in the recovery of menses, but also in achieving pregnancy. Moreover, a recent report by Demeestere et al., of a prospective randomized trial, with a median follow-up time of five years, showed that the GnRH agonist is not efficient in preventing chemotherapy-induced premature ovarian failure and has no influence on the future pregnancy rate (83). Therefore, temporary ovarian suppression with GnRH agonists during chemotherapy should not be considered as an alternative to embryo/oocyte cryopreservation or ovarian tissue freezing (84). In patients interested in fertility preservation, embryo and oocyte cryopreservation remain the first options to be proposed (85,86). Temporary ovarian suppression with GnRH agonists during chemotherapy should be considered in patients interested in ovarian function preservation only (e.g. in women concerned about the risk of developing treatment-induced premature ovarian failure but not interested in having a subsequent pregnancy). It is also an option for those interested in fertility preservation after cryopreservation procedures or when all other techniques are contraindicated or not available.

1.6.4. Cryopreservation of semen

Semen cryopreservation is a simple procedure that holds foremost importance for men who have cancer and who wish to preserve their reproductive options. The semen should be collected and frozen before the patient begins treatment for cancer, especially if the treatment involves chemotherapy or pelvic radiation. Testicular cancer is the most common malignancy in men of reproductive age (87). It is also the most common diagnosis which prompt men to cryopreserve their sperm. The prevalence of other diagnosis is outlined in Figure 4:

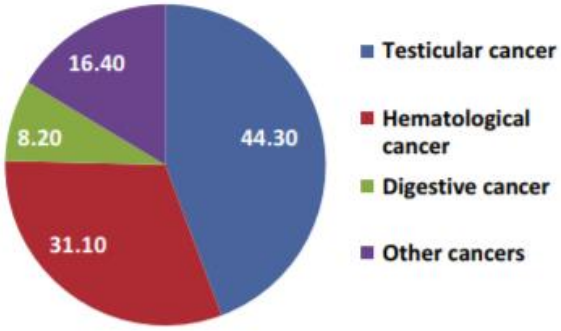

Figure 4. Types of cancer (%) in oncological patients who chose to cryopreserve semen. The "other cancers" category included bone sarcoma, thoracic cancer, and cancers of the prostate, mediastinum, mouth, brain, throat, lung, skin, and penis

Reprinted from: Kobayashi H, Tamura K, Tai T, Nagao K, Nakajima K. Semen cryopreservation as an oncofertility treatment in 122 Japanese men with cancer: A decade-long study. Reprod Med Biol. 2017;16:320–324.

Semen for sperm cryopreservation is generally obtained by masturbation. It is the conventional, simplest, and most noninvasive method. However, at the time of diagnosis, many cancer patients are inpatients, of which some men might be already quite ill and unable to produce a sample. In these cases, surgical sperm retrieval can be offered. In postpubertal men, vibratory stimulation and/or electroejaculation (EEJ) may be an option. Unfortunately, electroejaculation for patients with normal sensory status requires general anesthesia. If the patient is too sick to undergo anesthesia, bedside sperm retrieval with local anesthesia may be considered, involving percutaneous epididymal sperm aspiration (PESA), needle testicular sperm extraction (TESE), or testicular sperm aspiration (TESA). The age of the patient and pubertal status are also potential dilemmas. Management of prepubertal men in brief may involve testicular biopsy or even orchiectomy for spermatogonia recovery, and finally cryopreservation followed by transplantation/stimulation. This is still experimental (88,89).

After sperm retrieval, semen analyses are performed on all samples prior to cryopreservation. Semen parameters should be documented according to the WHO guideline. Sperm quality is defined by sperm count, sperm motility, and sperm morphology as well as freezability, for example the rate of post thaw viability of the semen. When semen is cryopreserved, a small subsample is frozen separately, thawed, and reanalyzed after the initial freeze. This test allows the post thaw survival to be determined as it can vary among individuals and even among different ejaculates from the same person.

The question of how many samples to freeze is determined by the sperm quality provided (90). This depends on the health of the patient and the type of cancer. It has been shown that the semen parameters of oncology patients before cancer treatment, both before freezing and after thawing, are worse than those of healthy donors (91,92). Hotaling et al. demonstrated that, prostate cancer had the best prefreeze total motile count (TMC) and lymphoid leukemia had the worst (92). Mean number of samples frozen is generally two.

Sperm quality also depends on the abstinence period between semen collections. In a study involving cancer patients, Agarwal et al. showed that semen collection cryopreservation after 24 to ≤48 hours of abstinence is a sufficient time span to reach post-thaw quality comparable to that after an abstinence of 48 to ≤72 hours or longer (93). This is substantial for cancer patients who have to initiate antineoplastic therapy as soon as possible, and beforehand leaving the best manageable quality of semen sample to cryopreserve.

Nevertheless, with IVF and ICSI, even the poorest samples are suitable to be frozen with high success rates. In a study of Meseguer et al. out of 186 cancer patients who banked sperm samples, approximately 15% actually made use of assisted reproduction technology (ART), resulting in 16 pregnancies (94).

Finally, it is important to mention again that samples should be obtained and frozen before initiation of gonadotoxic therapy. In patients who have already been treated with antineoplastic measures, or patients who regain spermatogenesis after therapy, there is a high risk of sperm production that is genetically or structurally affected (94).

2. OBJECTIVE

The objective of this study is to outline the various advances that have been made in oncofertility, as well as its current weak points. It should highlight the still existing gender disparity and the problem of availability of certain techniques, like ovarian tissue freezing. The study also discloses the prevalence of cancer patients who need and want to undergo oncofertility procedures in Split. Furthermore, it should raise awareness and inform physicians as well as patients about the numerous possibilities of FP in patients diagnosed with cancer. Finally, the study points out the positive impact of this new emerging and important field of medicine on cancer survivors' quality of life.

3. MATERIALS AND METHODS

3.1. Study design

This retrospective case-control study was conducted in the Department of Obstetrics and Gynecology of the University Hospital Split of the University of Split, School of Medicine. The data stems from a time frame of 5 years, 2013 to 2018 respectively.

3.2. Study Population

In this study 72 patients were included, 66 men who cryopreserved sperm and 6 women who cryopreserved oocytes or embryos. All patients made use of FP techniques because of a cancer diagnosis. Exclusion criteria was any other motive for cryopreservation. Eligible patients were identified using data from the files of the Obstetrics and Gynecology Department at the University Hospital of Split.

3.3. Materials

Medical data of eligible patients was retrieved from the Reproductive Medicine laboratory at the University Hospital Split. Following laboratory data was collected for each patient, if available:

1. Gender
2. Year of birth
3. Year in which cryopreservation took place
4. Clinical condition of the patient which motivated him/her to preserve fertility

3.4. Statistical Evaluation

All collected data were gathered in a Microsoft Office Excel sheet. Data analysis was conducted using the statistical software STATISTICA 12. In this study data has been reproduced in the form of tables, graphs and methods of descriptive statistics. The difference in count between men and women was calculated with the Chi-Square test and a linear trend analysis was customized. The significance level was determined to be $p < 0,001$.

4. RESULTS

In this study we collected data of 72 patients with cancer who underwent ART procedures in our department (Figure 5).

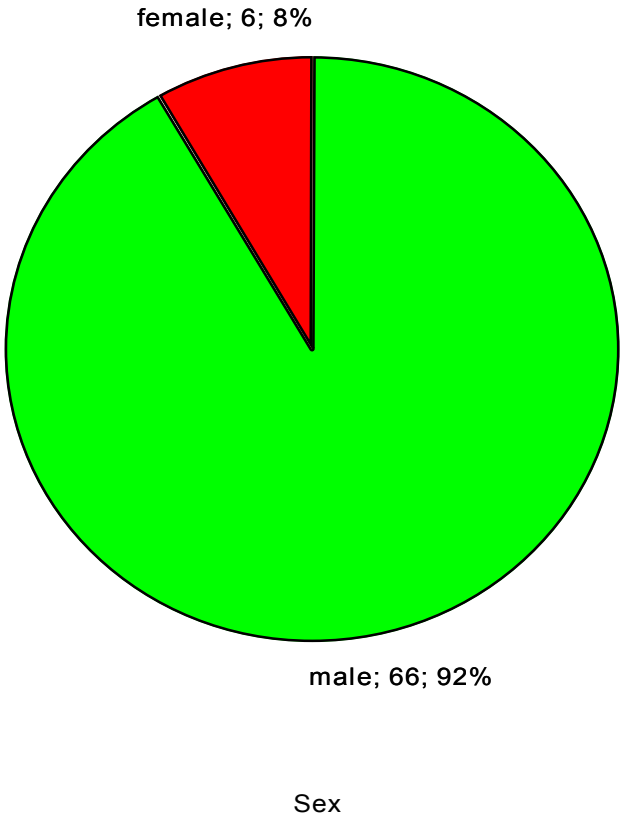

Figure 5. Gender of patients

This figure depicts the distribution of gender in our study population. While 92,5% of patients are male, only 7,5% are female (Chi square value 50,00; df=1; *p<0,001*).

The average age is 30 years with a standard deviation of ± 5,7. The variation of the average value is 18,99%, as calculated in table 1. This data shows that most of the patients using cryopreservation are young patients in their reproductive age.

Table 1. Descriptive Statistics

	N	Mean	Median	Q1	Q3	Std. dev. ±	Coef.var. (%)
Years of old	72	30.04	31	26	35	5,70	18,99

When analyzing the age distribution in Figure 2, the analysis shows that most patients in both groups (male and female) are 30 to 34 years old.

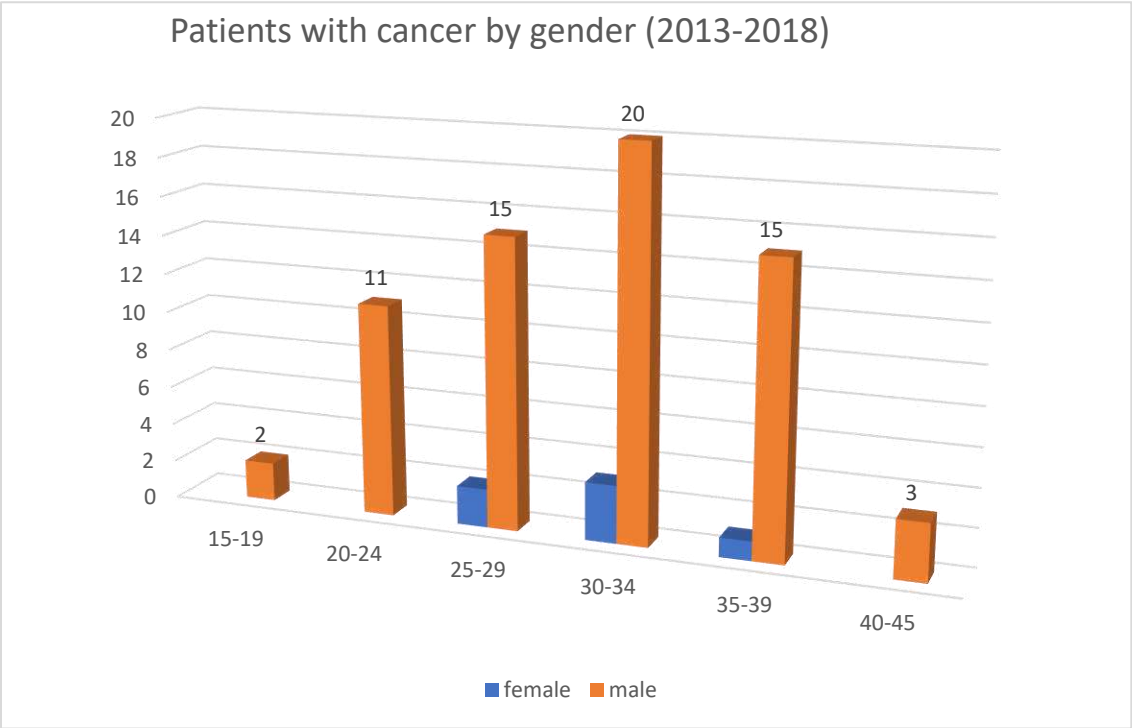

Figure 6. Distribution of patients by age and gender

Table 2. Type of cancer by gender

	Sex - male	%	Sex - female	%	Row - Totals
Testicular cancer	56	84.6%	0	0.0%	53
Breast cancer	0	0.0%	5	83.3%	5
Hodgkin Lymphoma	3	4.6%	0	0.0%	3
Sarcoma	2	3.0%	0	0.0%	2
Non Hodgkin Lymphoma	1	1.5%	0	0.0%	1
Acute Leukemia	1	1.5%	0	0.0%	1
Medulloblastoma	0	0.0%	1	16.7%	1
Colon cancer	1	1.5%	0	0.0%	1
Brain Tumor	1	1.5%	0	0.0%	1
Anal cancer	1	1.5%	0	0.0%	1
Totals	66	100.0%	6	100.0%	72

Most male patients undergoing ART have testicular cancer. Out of 66 patients included in the study, 56 present with this type of cancer. Most female patients have breast cancer. Out of our 6 female patients, 5 were diagnosed with this type of cancer.

Table 3. Patients with cancer undergoing FP procedures from 2013 to 2018

Cancer patients by years		
Year	**N**	**%**
2013	14	19.44
2014	10	13.89
2015	6	8.33
2016	21	29.17
2017	20	27.78
2018	1	1.39
Total	72	100.00

Table 3 demonstrates that in 2016 and 2017 more patients underwent ART procedures than in the 3 years before. The number climbed from 14 patients in 2013 to 20 patients in 2017.

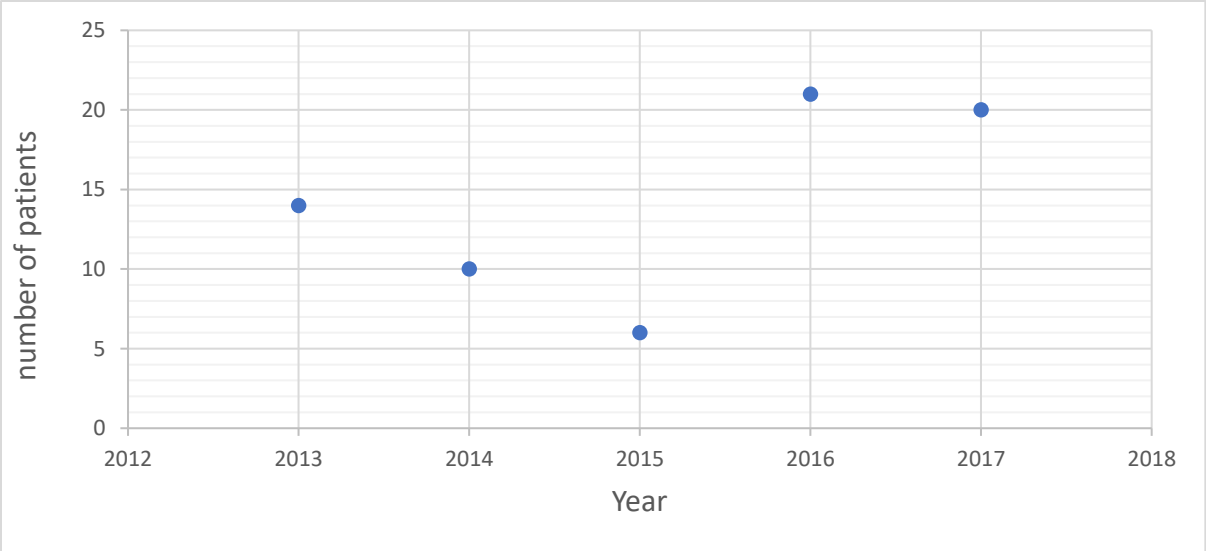

Figure 7. Patients' prevalence by year

Figure 7 points out how many cancer patients were treated for FP reasons in our department each year in the last 5 years. In 2013, 14 patients have been treated and in 2017 there have been 20 patients.

Table 4. Assumption of patients with cancer from 2013 to 2022

Time	Number of patients
2013	14
2014	10
2015	6
2016	21
2017	20
2018	**21***
2019	**23***
2020	**26***
2021	**28***
2022	**30***

*expected value by linear trend model

Table 4 illustrates the expectation of cancer patients undergoing ART procedures in the future, using a linear trend model. Calculated on basis of the patient count during the last 5 years, in 2022 we would expect to have around 30 patients.

5. DISCUSSION

In the past decade, the concept of oncofertility for cancer patients has been rapidly evolving. This has been the result of increased post-cancer survival worldwide and the great impact of fertility preservation (FP) on the quality of life of cancer survivors (95). Currently, however, as our study has proven, there is only a small number of young cancer patients who receives fertility preservation services. This owes to a range of factors. First, it was only possible to include a small study population, limited to the patients of the University Hospital of Split and our study can therefore not represent numbers globally. Data about the actual disease of patients was sometimes missing in the documents, so not all potential participants could be included. Also, certain techniques, like ovarian tissue banking, are not yet available in our hospital and patients undergoing those interventions are not taken into account. Generally speaking, number one reason for the restrained usage of FP in Split as well as internationally, seems to be a universal lack of knowledge. Both physicians and patients are not well informed about available procedures in reproductive medicine. Early referral by oncologists before initiation of chemotherapy and radiotherapy is an important step for success in fertility preservation strategies. Unfortunately, it has been shown that a lot of oncologists fail to counsel their patients adequately. However, oncofertility not only includes oncology and reproductive medicine but several other disciplines such as gynecology, urology, internal medicine, pediatrics, psychology and bioethics. It is therefore necessary to promote further education and continuous training in all these fields, to ensure the best cooperation and results in this upcoming branch.

Health networks are essential to improve coordination of care, and the strengthening of this coordination is a major challenge to improve the performance of the health system. Throughout the past 10 years, numerous international guidelines were published concerning anticancer treatments and fertility preservation. Such important guidelines were published by American Society of Clinical Oncology (ASCO), American Society for Reproductive Medicine (ASRM), European Society for Medical Oncology (ESMO), American Oncofertility Consortium (OC), International Society for Fertility Preservation (ISFP), Fertility Preservation Network FertiPROTEKT, American Academy of Pediatrics (AAP), and Association of Pediatric Hematology/Oncology Nurses (APHON) (96).

Nowadays embryo and oocyte cryopreservation are considered the golden standard for female cancer patients, and sperm cryopreservation for male cancer patients. A new and promising approach for females, especially prepubertal patients, is ovarian tissue freezing. While embryo CP is the first and most widely used option in the world, ovarian tissue banking is not universally available. Currently there are only a few medical centers with international

experience in performing ovarian tissue banking: about 100 worldwide and including 23 in France (95). Unfortunately, it is not yet available in Split.

Another issue in oncofertility seems to be the inequality in access between men and women. In our study 66 men made use of sperm banking while only 6 women underwent embryo or oocyte CP. This certainly owes to the fact that ART in women demands a far more complicated and time-consuming procedure, including COS and finally harvesting oocytes via transvaginal ultrasound. This is a painful procedure which might even require sedation or anesthesia. Moreover, women might not be aware of the potential fertility loss, while still being shocked by their diagnosis, and might also fear to delay cancer treatment or the procedure of ART itself. It has been reported that adequate counseling and assisted decision-making improve the number of women who choose to undergo fertility preservation treatments (97).

Our study suggests an incentive upward trend of FP methods. Excluding the data for 2018, which are not representative as they do not incorporate all patients who underwent or are still undergoing procedures and taking into consideration that our laboratory was only working for 2 months in 2015, we can see a slight but steady rise in the number of patients during the last 5 years. This also reflects in our linear trend analysis, which states that in 2022, 30 patients (16 more patients than in 2013) with cancer may be treated for FP reasons in our department in the University Hospital of Split.

Although the number of the patients who receive fertility preservation services has been increasing, another major factor, concerning female as well as male patients, is certainly the severe physical and emotional stress after cancer diagnosis. Having to make a decision with limited time, while still coming to terms with a potentially life-threatening diagnosis, can cause patients to feel overwhelmed. A poor-quality fertility discussion and inadequate information may add to this feeling of uneasiness (98). A survey on adult and pediatric oncology providers in the USA showed that the majority felt discomfort while discussing on fertility preservation, partly because of the lack of knowledge about options or places to refer, and that this discomfort hindered final referrals to specialists (99). It is therefore essential to inform all cancer patients thoroughly about current techniques and available medical centers, and to integrate them into systematic long-term follow-up. Lack of time and lack of knowledge are identified as the main barriers to the initiation of FP discussion and the training of healthcare providers remains a challenge until this day. It is crucial to further promote education in oncofertility measures in order to yield a higher number of patients undergoing procedures in the future.

However, it should be noted that the still limited use of these procedures may be due also to their high costs (100). A study of Katz et al. has shown that in America median per-

person costs ranged from $1,182 for medications only, to $24,373 and $38,015 for IVF. Within the timeframe of the study, costs were not significantly different for women who were successful in achieving pregnancy and women who were not (101). This puts an additional financial burden on cancer patient. First of all, it is not sure that ART procedures will be successful, second of all patients face an unsafe future being diagnosed with cancer. Undergoing such expensive treatment carries the risk of not paying off in the end.

Another potential hazard is the topic of FP in pediatric patients. Pivotal differences in the FP procedures offered to children and adults may affect the decision. In adults, the procedures available have proven efficacy and are less invasive. However, gonadal tissue cryopreservation in children is invasive, experimental, and carries a risk of reimplantation of cancerous cells. Although FP using established techniques may have positive outcomes and increase satisfaction in adults, this may not translate to the experiences of young children. Furthermore, the overwhelming feeling commonly reported among adult cancer patients making FP decisions, may be more extreme for parents, as they decide not for themselves but their child and as there is no guarantee of success (98).

In pediatric patients as well as in adults, there is still a lot of room and need for new techniques and procedures to best preserve fertility in the future. Several novel approaches are under development for fertility preservation in women and men. In vitro growth and maturation of follicles is a promising technique for female patients. Immature follicles, such as secondary, primary, or even primordial follicles, are harvested from ovarian tissue and cultured in vitro to produce mature oocytes (102). This technique would have the advantage of overcoming the risk of MRD following ovarian tissue cryopreservation. Another approach which has been successful in rodents, would be generating germ cells in vitro from pluripotent stem cells, although many technical and ethical issues need to be addressed for humans. New strategies for gonadal protection are also under investigation (103). One is the drug delivery system (DDS) to enhance the site selectivity and reduce the exposure of ovary, a nontargeted organ, to anticancer agents. The other is to administer the agents which reduce gonadal damage. For prepubertal male patients, testicular tissue cryopreservation is the method of choice to preserve fertility and offer reproductive options later on. In young boys however, testicular tissue contains sperm stem cells (SSC) but may not yet contain mature spermatozoa. Mature spermatozoa are the only cells though that can be used for contemporary fertility techniques. Future techniques include in vitro maturation of SSC into mature sperm cells for subsequent use in IVF/ICSI, or germ-cell transplant into native testicular tissue to allow fertility restoration. These techniques have been performed successfully in animal models but never in humans

(104). Maturation of SSC followed by IVF/ICSI has been shown to work remarkably well in mouse models in a study of Sato et al. in 2013, and the full differentiation of human SSC into mature sperm cells in vitro was recently demonstrated by Zhou et al. in 2016 (105,106). However, IVF/ICSI using human mature sperm cells derived from SSC in vitro has not been shown. Autologous testicular cell transplantation is an exciting potential option that has been used successfully with many animal models since 1994 (107). In 2012, a study of Herman et al. demonstrated the feasibility of testicular cell transplantation for restoring fertility in Rhesus macaque after undergoing bone marrow transplantation (108). Transplanting germ cells back into the gonads of a human patient after anticancer therapy to restore fertility potential has not yet been attempted. Among many technical challenges remaining is the task of purifying SSC populations effectively so that no malignant cells are reintroduced in the process.

Physicians as well as patients being not well acquainted with the subject of oncofertility, high costs, lack of knowledge and high-quality counseling, lack of sufficient long-term data on specific methods in humans, lack of reproductive medical centers worldwide and certainly also the lack of currently available procedures, result in many cancer patients not choosing to undergo fertility preservation. Hence, as endorsed by major international guidelines, it is crucial to promote the branch of oncofertility internationally, to provide services to the patients worldwide. Furthermore, we must improve the knowledge by long term follow-up of cancer patients undergoing ART and reduce costs to make the procedures more available for everyone in need. The final goal should be to counsel all cancer patients about the treatment-related loss of fertility and to assist them in taking decisions on fertility preservation to ensure their best quality of life after antineoplastic therapy.

6. CONCLUSION

1. Until this day, there is an uneven distribution in gender concerning the use of fertility preservation measures. While 92,5% of patients are male, only 7,5% are female. This stems from a combination of lack of knowledge concerning available procedures in women, high costs and the fear of the intervention itself.
2. The average age for cancer patients undergoing fertility preservation procedures is 30 to 34 years. This shows that most of the patients using cryopreservation are young patients in their reproductive age.
3. A majority of male patients undergoing ART have testicular cancer. Out of 66 patients included in the study, 56 were diagnosed with this type of cancer. Most female patients have breast cancer, meaning 5 female patients respectively.
4. If we consider the usage of ART during the last 5 years, we can expect an upward trend in the future, where more and more cancer patients will undergo interventions to secure their fertility.

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8. SUMMARY

Objectives: The objective of this study is to raise awareness and inform physicians as well as patients about the numerous different possibilities of fertility preservation in patients diagnosed with cancer. It should highlight the still existing gender disparity and the problem of availability of certain techniques, like ovarian tissue freezing. The study also discloses the prevalence of cancer patients who need and want to undergo oncofertility procedures in Split.

Materials and Methods: In this study 72 patients were included, 66 men who cryopreserved sperm and 6 women who cryopreserved oocytes or embryos in the University Hospital of Split. All patients made use of fertility preservation techniques because of a cancer diagnosis. Data was collected from patients' files of the Department of Gynecological Endocrinology and Human Reproduction from the last 5 years.

Results: In our study 92,5% of patients are male, and only 7,5% are female (Chi square value 50,00; df=1; $p<0,001$). This clearly outlines the predominance of male (66) over female (6) patients. The average age is 30 years with a standard deviation of ± 5,7. The variation of the average value is 18,99%. When analyzing the age distribution, the analysis shows that most patients, male and female, are 30 to 34 years old. Most male patients undergoing ART have testicular cancer. Out of 66 patients included in the study, 56 present with this type of cancer. Most female patients have breast cancer. Out of our 6 female patients, 5 were diagnosed with this type of cancer.

Conclusion: For oncofertility to function and achieve the best possible results, it is necessary to properly educate all physicians involved in the care of the patients. The availability of procedures needs to be improved internationally. More women diagnosed with cancer should be able to have recourse to fertility preservation. It is of great significance to further invest in this branch of medicine in order to improve safety and physician's performance on existing methods, as well as revealing new procedures.

9. CROATIAN SUMMARY

Naslov: Onkofertilitetni postupci Zavoda za ginekološku endokrinologiju i humanu reprodukciju, Klinički bolnički centar Split

Cilj: Cilj ovog istraživanja je povećati svijest i informirati liječnika i pacijente o brojnim mogućnostima očuvanja plodnosti kod oboljelih od malignoma. Valja istaknuti i dalje postojeće rodne nejednakosti i problem dostupnosti određenih tehnika, kao što je zamrzavanje tkiva jajnika. Studija također otkriva učestalost bolesnika s karcinomom koji trebaju i žele proći oncofertility postupke u Splitu.

Materijali i metode: U ovu studiju su uključena 72 pacijenta, 66 muškaraca koji su kriopohranili svoje sjeme i 6 žena koje su kriopohranile svoje zametke ili jajne stanice. Svi su to učinili zbog maligne bolesti a u smislu očuvanja kasnije plodnosti. Podaci su prikupljeni od pacijenata datoteke Odjela za ginekologiju endokrinologiju i humanu reprodukciju od zadnjih 5 godina.

Rezultati: U našem istraživanju 92,5% pacijenata su bili muškarci i samo 7,5% su bile žene (Hi-kvadrat test 50,00 df=1 $p<0,001$). Navedeno pokazuje izrazitu predominaciju muških nad ženskim pacijentima. Prosječna dob ispitanika je 30 godina sa standardnom devijacijom ±5,7. Varijacija prosječne vrijednosti je 18,89%. Analiza dobne distribucije pokazala je da je većina i muških i ženskih pacijenata između 30-34 godine starosti. Većina muškaraca koji su kriopohranili svoje sjeme bolovala je od tumora testisa. Od njih 66 uključenih u istraživanje čak njih 56 imalo je tumor testisa. Većina žena bolovala je od tumora dojke. Od njih 6 uključenih u studiju, pet ih je imalotumor dojke.

Zaključak: Za onkofertilitetne postupke da bi funkionirali i postigli što bolje rezultate neophodno je adekvatno educirati sve liječnike uključene u rad s ovimpacijentima. Dostupnost postupaka treba poboljšati na međunarodnoj razini. Više žena dijagnosticiran rak bi trebao biti u mogućnosti obratiti se očuvanje plodnosti. Od velikog značaja za daljnji razvoj ove grane je povećati sigurnost za pacijente te uspješnost postojećih metoda uz razvoj novih onkofertilitetnih postupaka.

10. CURRICULUM VITAE

Curriculum Vitae

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Education

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