Basic and clinical approaches for fertility preservation and restoration in cancer patients

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ABSTRACT

As gonadotoxic side effects of anti-neoplastic treatments can result in infertility, gamete cryopreservation is routinely offered to patients as the strategy to preserve their fertility. However, there are many cases where gold standards cannot be applied, as is the case for prepubertal cancer patients and others unable to produce gametes or their precursors at the moment of diagnosis. With an increasing number of cancer survivors in our society, strategies using either cryopreserved gonadal tissue or stem cells have been developed to allow cancer survivors to accomplish fatherhood and recent advances in the field have increased public interest. In this review, we discuss the latest updates in fertility preservation from a basic and a clinical point of view.
Key words: Fertility preservation; Ovarian cortex cryopreservation; Germ cell transplantation; Testicular tissue grafting; In vitro maturation; In vitro differentiation.
WHAT IS FERTILITY PRESERVATION?

Fertility preservation (FP) is an emerging field that offers treatments aimed at protecting future reproductive ability for individuals [1]. Although nowadays there is an emerging demand on FP treatments from women that desire to postpone their motherhood for several social reasons, oncologic patients and others subjected to treatments that may compromise their future reproductive chances represent the main group of patients where there is a medical indication for FP.

Significant advances in treatments have turned cancer into a chronic pathology in many cases. However, chemotherapy and radiotherapy to fight neoplastic cells (see Glossary) usually have gonadotoxic side effects that can result in infertility. Thus, with current child cancer survival rates reaching close to 80% or even higher [2], the reproductive options of cancer survivors are an important issue to bear in mind. Therefore, cryopreservation of gametes and embryos is usually offered before starting gonadotoxic treatments as the main strategy for FP.

Nevertheless, these strategies are not possible when patients are unable to produce their own gametes, as is the case for prepubertal patients, or in women when ovarian stimulation is not indicated because it would interfere with the patient’s treatment plan.

In this regard, there is a high social and scientific interest in the development of alternative strategies that allow these patients to preserve their fertility. Although still in experimental stage, some of these strategies are already applied in the clinic, as is the case for ovarian cortex cryopreservation (OTC) and re-transplantation once the cancer has been cured. Other strategies, however, are still in research stage, as happens with the use of either spermatogonial stem cells (SSCs) [3] or induced pluripotent stem cells (iPSCs) [4] as a source of mature gametes.

In this comprehensive review, we will highlight and discuss the most recent advances in FP, with special focus on those that may become a clinical option in the near future.
WHICH PATIENTS ARE CANDIDATES FOR FERTILITY PRESERVATION?

Adverse effects of antineoplastic therapies on the reproductive organs result from a direct effect from surgery or radiotherapy on the hypothalamic–pituitary–gonadal axis, or damage to the gonads due to chemo and radiotherapy. Although gonadotropin deficiency caused by damage to the hypothalamic–pituitary–gonadal axis can be restored by the exogenous administration of gonadotropins, damage in the gonads may result in impaired pubertal development, hormone insufficiency, fertility problems and sexual dysfunction [5]. Table I summarizes the main factors that are likely to affect the reproductive outcome of cancer patients and the biotechnological strategies aimed to improve it (Table I).

In females, chemotherapy can induce apoptosis of primordial follicles, leading to a reduction of Anti-Müllerian Hormone (AMH) levels and accelerating the recruitment of surviving primordial follicles, resulting in burnout [6] of the ovarian reserve and premature ovarian failure. In males, spermatogenesis is extremely sensitive to the damaging effects of alkylating agents and thus oligozoospermia or azoospermia is a common outcome in cancer patients [7]. Importantly, the precise combination of chemotherapeutic drugs and the frequency and duration of administration determine their gonadotoxic effects. Also, since ovarian reserve decreases with age, similar amounts of chemotherapy and /or radiotherapy may have even more gonadotoxic effects in older women with an already decreased ovarian reserve [8-10].

Considering this, as long as FP does not imply a delay in treatment initiation, and considering the risk-benefit of an ovarian stimulation depending on the age and gonadotoxic risk in the case of females, all cancer patients able to produce gametes at the moment of diagnosis should be advised to cryopreserve them. Nevertheless, defining clear candidates for more aggressive or experimental FP techniques is a challenge for clinicians [11, 12]. In females, the Bologna criteria recently introduced the assessment of AMH levels and antral follicle count as
selection criteria for OTC. Based on these criteria, the 25th percentile of both markers could represent an acceptable lower threshold for counseling FP to women regardless of their age. On the other hand, only female cancer patients under 35 years between the 5th and 25th percentiles should be selected for FP, and below the 5th percentiles all patients should be excluded considering the costs/benefits ratio of the technique [13]. In fact, the AMH level at cancer diagnosis has been proposed as a long-term predictor of ovarian function allowing a better assessment of the chemotherapy-induced ovarian failure risk [14-16]. Nevertheless, besides the thresholds of ovarian reserve markers, patient’s age is also a main limitation to offering FP to women since after the age of 36 oocyte cryopreservation leads to significantly poorer outcomes[17]. Therefore, specific predictive models should be developed depending on patient’s age and malignancy.

Regarding the gonadotoxic risk depending on the chemotherapeutic drugs administered to girls, the Edinburgh selection criteria advise FP counseling when there is a calculated risk of ovarian failure of over 50% [11], whereas globally for all women, the FP expert committee defends a lower cut-off point, (risk over 30%) [18]. Nevertheless, due to interpatient variability and insufficient evidence, it is not possible to make accurate recommendations regarding risk stratified surveillance strategies based on either dose thresholds or age at treatment [19]. Therefore, if the initial purpose of a treatment is curative, realistic good prognoses could facilitate decision making. Regarding this, Tables II and III summarize the main published data of gonadotoxic risk in males and females, and suggest cut-off thresholds for infertility risk associated to each agent and dose (Tables II and II).

Of note, in the case of prepubertal children, special attention should be given to practical and ethical challenges for patients and their families. In this context, close coordination between oncological, surgical and gynecological/urological teams is highly desirable to harvest ovarian/testicular tissue for FP at the same time that other already programmed surgical procedures (venous central line insertion or therapeutic surgery) take place, in order to reduce

THE GOLD STANDARDS OF FERTILITY PRESERVATION IN FEMALE CANCER PATIENTS: OOCYTE AND EMBRYO VITRIFICATION

For female cancer patients at reproductive age, both embryo and oocyte cryopreservation are the gold standard FP methods (Figure 1) [20]. Importantly, controlled ovarian stimulation protocols with GnRH antagonists can be initiated in the luteal phase [21], ensuring the oocyte collection within two weeks, thus avoiding further delays in the administration of oncologic treatments. Also, adverse effects of estradiol levels during stimulation in women with hormone-sensitive breast cancer can be modulated by the administration of aromatase inhibitors (letrozole) or selective estrogen receptor modulators (tamoxifen). Alternatives aimed to limit the ovarian damage such as ovarian transposition (oophoropexy) when pelvic radiation therapy is performed have also been described, but the use of ovarian suppression by gonadotropin-releasing hormone (GnRH) analogs is discouraged due to the insufficient evidence regarding its effectiveness [22].

Oocyte cryopreservation is an already established method in assisted reproduction technology (ART) that can be offered to preserve fertility of cancer patients with mature oocytes. Vitrification, which uses high concentrations of cryoprotective agents and fast cooling rates to avoid cellular damage by ice-crystal formation, has become a landmark in FP [23]. In contrast with the low pregnancy rate per thawed oocyte of less than 3% shown by slow freezing [24], vitrification clinical outcomes in cancer patients are comparable with those of fresh oocytes, with reported survival rates of 92.3%, fertilization rates of 76.6%, and a mean number of 1.8 ± 0.7 transferred embryos [25].

Alternatively, embryo cryopreservation represents the first established technique to preserve fertility since the first reported pregnancy in 1983 from a cryopreserved embryo [26]. Although
slow-freezing of embryos is currently employed in many clinics, a recent meta-analysis suggested that vitrification/warming is superior to slow-freezing/thawing with regard to clinical outcomes (low quality of the evidence) and cryosurvival rates (moderate quality of the evidence) for oocytes, cleavage-stage embryos and blastocysts [27]. On the other hand, with survival rates of vitrified embryos ranging from 35% to 90% [28] and live birth rates from 22% to 45% per embryo transfer, the outcomes after vitrified/warmed embryo transfer in cancer patients undergoing FP show similar rates to those obtained with fresh embryos in non-cancer patients [29]. However, since sperm is required, it links further fertility to the male partner, thereby excluding single adult women and pre-pubertal cancer patients from the oncologic population that can benefit from it. Also, embryo vitrification has ethical issues that restrict this technique in some countries [27].

Unfortunately, these strategies cannot be offered to patients for whom there is no time for ovarian stimulation, which is the case for women with hematological diseases or aggressive malignancies, where the first cycle of chemotherapy must not be delayed, or for pre-pubertal patients for whom ovarian stimulation is not feasible. For these patients, alternative strategies are gaining importance.

**OVARIAN TISSUE CRYOPRESERVATION AS AN ALTERNATIVE OPTION FOR FERTILITY PRESERVATION IN FEMALE CANCER PATIENTS.**

OTC and re-transplantation has been proven to be a suitable FP alternative in patients at high risk of ovarian failure when there is no time to stimulate them and retrieve mature oocytes (Figure 1). However, despite its success with 86 reported live births and 9 ongoing pregnancies [30], including one live birth from a woman who cryopreserved ovarian cortex when she was a child [31], it is still considered experimental. Therefore, OTC and re-transplantation is only performed in a few centers worldwide [32].
In contrast to embryo and oocyte vitrification, OTC does not require ovarian stimulation, thus interfering minimally with the patient’s treatment plan. By using this approach, quiescent primordial follicles, already present in ovaries at birth and the main component of the ovarian reserve, are cryopreserved, hence making this technique suitable for patients of all ages. Each quiescent follicle, composed by a dormant oocyte surrounded by a unique layer of flattened granulosa cells, maintains the ability to begin its development and to reach the antral stage before ovulation [33].

Ovarian tissue can be retrieved either by ovarian biopsy or oophorectomy, after which the ovarian medulla is removed and the cortex containing the primordial follicles is cryopreserved. Although several studies have evaluated vitrification as an alternative to slow freezing [34] and live births have been reported after human ovarian tissue vitrification [35], slow freezing is the elected technique in most FP programs [36].

Almost all reported pregnancies have been achieved after grafting ovarian tissue back onto the ovary, broad ligament or adjacent pelvic tissue (orthotopic sites) [37]. However, delivery of twins following heterotopic grafting has also been achieved [38].

Of note, since ovarian cortex is grafted as an avascular transplant, follicular depletion occurs during the first days after grafting due to ischemic damage [39]. Furthermore, since leukemic cells can cause recurrence of the disease when reintroduced into severe combined immunodeficient mice [40], OTC is restricted to malignancies that do not affect the ovary directly [41]. In these cases, histological analysis may be helpful but not enough to rule out the possibility of contamination of malignant cells within the tissue.

**FUTURE DIRECTIONS IN FEMALE FERTILITY PRESERVATION: IN VITRO MATURATION OF PRIMORDIAL FOLLICLES**
To date, the available techniques for FP associate restrictions and limitations that should be solved to allow safe and successful methods to achieve motherhood after cancer regardless of the disease, age at diagnosis or patient’s social status. Having all these factors in mind, in vitro maturation (IVM) of follicles and oocytes (Figure 1) is being developed during the last decade. In fact, IVM and subsequent oocyte vitrification are already being offered together with OTC to increase FP options as immature oocytes can be successfully collected by aspiration from small antral follicles during tissue manipulation for OTC [42-44]. Although further investigation is needed, these in vitro matured oocytes seem to retain their developmental potential [44, 45] as clinical pregnancies have been achieved [46]. Moreover, growth of human oocytes from primordial follicles has been attempted by incubation of small pieces of ovarian cortex [47], isolation and culture of follicles [48], or combination of both [49]. Using a two-step protocol, human primordial follicles have been able to grow in vitro, differentiate, produce steroids and maintain normal oocyte morphology [49]. Also, v-shaped microwell plates have allowed maintenance of 3D follicular architecture while promoting growth and differentiation of human follicles up to antral formation after 28 days in vitro [49]. Although only non-functional human Metaphase II oocytes could be obtained by this method, some important issues have been solved by using 3D artificial ovaries, providing an artificial environment to support survival and growth of isolated follicles [50]. Also, it has been reported that a transplantable artificial ovary consisting of a 3D printed scaffold seeded with isolated mice follicles was able to restore fertility in animals [51].

THE GOLD STANDARD OF FERTILITY PRESERVATION IN MALE CANCER PATIENTS: SPERM BANKING

Sperm cryopreservation is an effective method routinely performed in fertility clinics since the first successful human pregnancy with frozen/thawed sperm was achieved in 1953 [52]. The
storage of sperm in liquid nitrogen is an effective, easy, and low-cost strategy for FP with reported fertilization rates ranging between 60% and 93% [53]. Also, it has proven successful for long-term cryopreservation, with a report of one healthy live birth using sperm samples cryo-stored for 40 years [54]. Therefore, cryopreservation of sperm has been the gold standard for FP in male cancer patients prior to a potentially gonadotoxic exposure (Figure 2).

Testicular volume over 10mL [55] and presence of sperm in urine are helpful tools to assess the sexual maturation of boys in order to offer sperm banking [56]. However, fever, hypermetabolism, nutritional alterations commonly associated with cancer, and endocrinopathies in the case of Hodgkin lymphoma, can negatively affect sperm production [57]. Moreover, sperm banking is not an option for prepubertal patients unable to produce sperm due to physiological immaturity but also to challenges in obtaining a sperm sample from emotionally and psychologically immature adolescents. Consequently, alternative therapies to preserve fertility in these patients are currently under investigation.

**CRYOPRESERVATION OF TESTICULAR TISSUE AS AN ALTERNATIVE OPTION TO PRESERVE THE FERTILITY OF MALE PATIENTS**

SCCs are the resident stem cells within the testes [58], responsible for the maintenance of spermatogenesis during adulthood. Due to their potential to restore spermatogenesis, there is a great interest in the use of SSCs as a tool for FP in patients unable to generate sperm, as can be the case for prepubertal boys (Figure 2). Indeed, according to a recent survey from the Oncofertility Consortium at Northwestern University ([http://oncofertility.northwestern.edu](http://oncofertility.northwestern.edu)) [59], there are currently at least 16 health centers around the world, seven of them in Europe [3], offering cryopreservation of testicular biopsies containing SSCs to prepubertal oncological patients. Most centers perform slow freezing, although vitrification has demonstrated to be effective in preserving human spermatogonia as well[60, 61].
Among the strategies designed to restore spermatogenesis in cancer survivors using immature cryopreserved testicular tissue, SSC transplantation is probably the most promising approach [62]. In the mouse model, transplantation of testicular cell suspensions containing SSCs into the seminiferous lumen of germ cell depleted hosts results in full restoration of spermatogenesis by colonizing SSCs, producing sperm that is able to give rise to healthy offspring [63]. Moreover, this technique has been applied to other animal models, including non-human primates, with successful production of sperm able to fertilize oocytes, even after long-term tissue cryopreservation [64]. Nevertheless, in vitro propagation of SSCs may be a necessary step before transplantation. Although long-term in vitro propagation of mouse SSCs has been reported by several groups [65], to date, approaches for in vitro propagation of human SSCs [66, 67] have demonstrated to allow only short-term survival of human SSCs in vitro [68].

Another promising strategy to restore spermatogenesis in cancer survivors is autologous immature testicular tissue grafting. This technique was originally described in a report where the engraftment of small pieces of prepubertal testicular tissue under the skin of immunosuppressed castrated host mice resulted in the production of sperm that could be retrieved for downstream ART applications [69]. This technique has also demonstrated its feasibility giving rise to sperm [70] and even healthy offspring in other species, including non-human primates [71]. However, xenografts from donor species with higher than average daily sperm production such as pig, goat and human into mice hosts show limited survival of the engrafted tissues due to insufficient vascularization causing ischemia [72]. Thus, although data suggest that grafts from prepubertal tissue show better resistance to ischemia due to the quiescent nature of the tissue, so far, human testicular xenografts have not been able to develop beyond the spermatocyte stage [73, 74].

The promising results obtained from animal models, together with an experience of more than 15 years cryopreserving immature testicular biopsies from prepubertal patients, encourage
further research in order to allow restoration of fertility in azoospermic men. Until then, cryopreservation of testicular tissue is still an experimental procedure that should be offered only to patients with a high risk of acquired azoospermia unable to produce sperm at the moment of diagnosis [3].

**FUTURE DIRECTIONS IN MALE FERTILITY PRESERVATION: IN VITRO SPERMATOGENESIS**

*In vitro spermatogenesis* is a third approach that has gained interest as a possible source of sperm from SSCs. Most efficient strategies to achieve the production of sperm from mouse SSCs comprise 3D co-cultures of both somatic and immature germ cells [75, 76]. Furthermore, cytocompatible scaffolds made from de-cellularized human testicular tissue may be used to test the potential of human SSCs to mature in vitro on a niche that mimics the extracellular matrix [77]. However, up till now, only an organotypic culture of mouse testicular tissue maintaining the cellular interactions and tissue integrity, has allowed production of mature and functional sperm able to be used in intracytoplasmic sperm injection (ICSI) [78, 79]. By culturing small pieces of newborn mice testicular tissue at 34ºC in the liquid-air phase in a medium containing KnockOut serum replacement, spermatids and sperm were retrieved after three weeks and used for ICSI, giving rise to healthy offspring. However, although this technique has been replicated [80, 81], a study where this organotypic culture was adapted to prepubertal human tissue, with modifications in media composition, indicated a blockade in maturation of spermatogonia accompanied by a progressive loss of germ cells along time, even though maturation of the somatic cells into a postpubertal phenotype was observed [82]. This observation supports a recent study where major differences were found in the differentiating response of spermatogonia to gonadotropins between monkeys and mice, indicating that functions of genes established to govern spermatogonial differentiation in the mouse may not necessarily translate directly to the primate testis [83].
GAMETES IN A PETRI DISH: ADVANCES IN OBTAINING GAMETES IN VITRO FROM PLURIPOTENT CELLS AND TRANSDIFFERENTIATION FROM SOMATIC CELLS

The possibility of reprogramming almost any cell type into iPSCs and differentiate them into germ cells, make them a promising source of functional gametes even when patients are unable to store gametes/gonadal tissue before the initiation of a gonadotoxic treatment (Figure 3) [4].

The potential use of pluripotent stem cells as a source of gametes was first reported in 2003 in mouse embryonic stem cells (ESCs) when oocyte-like structures capable of parthenogenic activation and forming pseudo-blastocysts were spontaneously generated [84]. However, the real functionality of in vitro produced gametes was recently demonstrated with the generation of viable offspring from sperm and oocytes derived from mESCs and iPSCs [85, 86]. Based on the unique potential of the embryonic epiblast to give rise to primordial germ cells (PGCs) in vivo, authors designed a two-step induction protocol for the formation of germ cells in vitro: first, naïve mouse ESCs and iPSCs were cultured to trigger their differentiation into epiblast-like cells (EpiLCs). In a second induction step, EpiLCs were further 3D-cultured within embryoid bodies and exposed to a cocktail of growth factors and cytokines to trigger the specification of PGC-like cells (PGCLCs) that expressed key PGC markers and showed signs of epigenetic reprogramming. Moreover, PGCLCs were able to differentiate either into sperm or oocytes when transplanted into the mouse testicular lumen or under the ovarian bursa in reconstituted mouse ovaries, respectively, giving rise to healthy offspring upon ART. The same group showed how ectopic expression of key transcription factors downstream of bone morphogenetic protein signaling (Prdm1, Prdm14 and Tfap2c) in pre-induced mouse EpiLCs resulted in a PGCLC phenotype as well [87], demonstrating for the first time a reliable strategy to produce functional gametes in vitro from pluripotent cells. More recently, another report
has identified the pluripotency-related gene Nanog as a master regulator of PGCLC specification from EpiLCs in vitro [88].

The success of the reports described above in the mouse model inspired others who applied a very similar approach on human ESCs and iPSCs [89-91]. In three studies, authors achieved the formation of a human PGCLC phenotype upon exposure of pre-induced mesoderm cells to a cocktail of growth factors based on the one previously used in mouse studies. These works highlighted the mesodermal origin of human PGCLCs from epiblast cells. Although human PGCLCs displayed a signaling network for germ line specification very similar to the one described for mouse, results from one of these studies also highlighted the important role of SOX17 in human germ line specification as the master regulator for human PGC specification [89, 92]. However, compared with mouse studies where PGCLCs were matured into functional gametes by transplanting them into mouse gonads, further maturation of human PGCLCs obtained in vitro was not investigated.

Two recent studies have overcome the need to transplant immature PGCLCs into an animal host to achieve their complete maturation and to form functional gametes in vitro [93, 94]. In both reports, authors also followed the two-step induction of PGCLCs from EpiLCs. However, instead of transplanting them into a mouse gonad, testicular somatic cells from mutant mice lacking germ cells were used to co-culture mouse PGCLCs. After two weeks of PGCLC/testicular cell co-culture with FSH, Testosterone and bovine pituitary extract, 14% of PGCLCs matured into spermatid-like haploid cells capable of generating healthy offspring upon ICSI [93]. With a similar approach, mouse PGLCs were aggregated with fetal ovarian cells and sequentially cultured in media designed to trigger differentiation, growth and maturation of follicles. As a result, follicle-like structures released from the aggregates formed Metaphase II oocytes that were fertilized and gave rise to healthy offspring [94]. Altogether, these new achievements have caused a breakthrough since they avoid the need of an animal host to mature functional
gametes. Nevertheless, the need for gonadal somatic cells to support the in vitro maturation of PGCLCs highlights the importance of the niche to drive the correct maturation of gametes.

From an alternative point of view, based in previous reports investigating the role of evolutionary conserved germ cell RNA-binding proteins such as the DAZL family and VASA [95-98]. A recent study opened a new interesting strategy for the derivation of human gametes by transdifferentiation of somatic cells (Figure 3) [99]. By the ectopic expression of six genes, human male somatic cells were transdifferentiated in vitro into a meiotic germ cell-like phenotype with ability to form haploid cells and undergone epigenetic reprogramming towards an oocyte-like epigenetic profile. Also, transdifferentiated cells were able to colonize the seminiferous tubules of germ cell depleted mouse testes in vivo. However, the need for genetic modification and the low efficiency of the protocol make this approach difficult to translate to the clinic at present.

**CONCLUDING REMARKS AND FUTURE PERSPECTIVES**

Despite the success of sperm freezing and oocyte/embryo vitrification to preserve the fertility of oncologic patients, patients unable to produce functional gametes at the moment of diagnosis cannot benefit from these strategies and thus new avenues are currently being developed. Nevertheless, despite great advances in the last decades, there is still a long way to go (see Outstanding Questions Box).

In the female counterpart, OTC and transplant is currently being performed in an increasing number of health centers. Alternatively, IVM of primordial follicles within ovarian cortex may be an alternative strategy to produce functional Metaphase II oocytes avoiding the risk reintroducing malignant cells. Nevertheless, current challenges for IVM of primordial follicles include tailoring of culture environments to match the physiological needs, the maintenance of
cell-cell communication and signalling during culture, the evaluation of the genetic, epigenetic status, and eventually, the functionality of in vitro-derived mature oocytes.

On the male side, the use of preserved testicular tissue to restore the fertility of males is still considered in a research stage since it has not been demonstrated to be useful in humans yet. Since transplantation of human SSCs into germ cell-depleted mouse testes results in colonization of the seminiferous epithelium, but fails to restore spermatogenesis because of phylogenetic distance between species [100], there is a need for appropriate models for spermatogenic restoration in humans. Also, due to the relatively low colonizing efficiency of SSCs and the limited size of prepubertal testicular biopsies, in vitro propagation of SSCs is a necessary step prior to perform transplantation in humans [65, 67, 68, 101, 102]. Moreover, considering that up to 21% of leukemias may present testicular infiltration of malignant cells [103], cell sorting can be a tool for the elimination of cancer cells prior to transplantation [104]. However, with the absence of reliable markers to isolate pure SSC populations from human testicular samples [65, 101, 102], in vitro propagation of human SSCs could be a useful strategy to both enrich SSCs and eliminate contaminating malignant cells. Alternatively, supplementation of testicular grafts with either vascular endothelial growth factor in order to prevent ischemia [105], or antiapoptotic factors [106], as well as the search of alternative locations such as intra-testicular grafting in order to mimic the temperature of testes [74] may improve the success of testicular grafting [107].

On the other hand, despite the important advances in different in vitro approaches such as the testicular organotypic culture, and strategies for gamete differentiation using either iPSCs or somatic cells as a source, their reproducibility using human tissue remains unclear. Especially, meiosis and later steps of gamete formation are tightly regulated by the niche and thus, a better understanding of the fine regulation of the human gonadal niche is mandatory for the improvement of in vitro spermatogenesis, iPSC differentiation and even transdifferentiation [108]. In this regard, bioengineering technologies such as the application of extracellular
matrix-based hydrogels to form scaffolds, or the use of microfluidics and nanoparticles to improve the delivery of growth factors, may also provide support for mimicking the gonadal niche and achieving the maturation of in vitro derived germ cells (reviewed in [109-111]). Nevertheless, due to the essential role of gametes to transmit genetic and epigenetic information between generations, assessment of safety and functionality of in vitro generated gametes is mandatory prior to their possible clinical translation.

Ultimately, the improvement of strategies for the maturation of germ cells in vitro, combined with new molecular techniques for gene editing can even be the next scientific keystone for the eradication of genetic diseases such as cancer related mutations in the offspring of cancer survivors [112-114]. However, ethical precautions are necessary for the application of this revolutionary technology. Interventions on the human genome should be admitted only for preventive, diagnostic or therapeutic reasons, therefore avoiding their use for eugenic goals.

Taken together, important progress has been achieved in the last decades towards new avenues to improve the life quality of cancer survivors by allowing them the chance of forming a family. Nevertheless, there is still a long way to go, so the combined efforts from both clinicians and scientists are necessary to in order to convert this goal in a reality.

ACKNOWLEDGEMENTS

This work was supported by a private donation of the Celtic Submari - Villareal C.F. to Hospital Universitario y Politécnico La Fe intended to promote the scientific research on fertility preservation in child with cancer, and a AES project grant (PI16/00931) conceded by the Instituto de Salud Carlos III to MMA.

AUTHOR CONTRIBUTIONS
JVM and MMA conceived this work. All listed authors contributed to specific sections of the manuscript according to their expertise. EG and AP revised and approved the final version of the manuscript.
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**TEXT BOXES**

**BOX 1: ARE THERE STEM CELLS IN THE HUMAN OVARY?**

It is generally accepted that female mammals are born with a finite number of germ cells (ovarian reserve) that decreases during lifetime [115]. However, this dogma was questioned when a revolutionary report postulated the existence of stem cells in postnatal mammalian ovaries able to replace atretic follicles by neo-folliculogenesis [116]. In order to test this hypothesis, ovarian fragments from wild type mice were transplanted into the ovarian bursal cavity of GFP mice, resulting in the formation of chimeric follicles [116]. The same group also suggested bone marrow as the putative source of ovarian stem cells based on the formation of chimeric GFP follicles upon transplantation of either bone marrow or peripheral blood from GFP mice into chemotherapy-ablated wild type females [117].

After an intense scientific debate, a parabiosis experiment between wild type and GFP mice demonstrated the absence of GFP oocytes in wild type females [118]. Transplantation of bone marrow from a GFP donor into sterilized wild type females also failed to demonstrate the presence of oocyte chimeras, suggesting that the recovery of ovulation in chemically sterilized mice could be explained by incomplete depletion of endogenous follicles.

In 2009, a new report [119] reactivated the debate claiming the in vitro derivation of germ stem cells from Mouse vasa homolog (Mvh) positive cells isolated from mouse ovaries. In vitro cultures of these cells expressed pluripotency-associated markers as well as early germ cell markers. More recently, the isolation and culture of Mvh/VASA germ cells from both mouse and human ovaries lead to spontaneous formation of oocyte-like cells able to complete meiosis in vitro [120]. Derived cells were further labelled with GFP and transplanted either into mouse ovaries or co-cultured with disassociated human ovarian cortical tissue that were transplanted into immunosuppressed mice, resulting in the formation of GFP oocytes able to form healthy embryos. However, another recent report from a consortium of independent
groups replicating the same experimental approach did not support these results [121].

Nevertheless, it has been reported that the number of primordial follicles within human ovaries is increased after exposure to certain chemotherapeutic regimens, suggesting that certain stress conditions may trigger the formation of new follicles [122].

Taken together, despite the implications neo-folliculogenesis within the human ovary may have in the design of strategies to restore the fertility of infertile women without ovarian reserve [123], the existence of ovarian stem cells in the human ovary is not fully supported and further research is needed in order to solve this scientific debate.


**BOX 2: GENOME EDITING IN HUMAN GAMETES**

The clustered regularly interspaced repeats (CRISPR) is a molecular defense mechanism in prokaryotes against viruses. The system is formed by an RNA palindromic sequence (CRISPR) and an endonuclease protein (Cas9) forming a complex that targets a specific region in the foreign viral DNA, generating a double-strand cleavage that is eventually repaired either by **homology-directed repair** or **non-homologous end-joining** pathways. Therefore, this highly specific mechanism has become a powerful tool for the correction of mutations in desired loci of human cells [89].

Gene editing via CRISPR/Cas9 has been demonstrated in many mammal cells, including *in vivo* animal models [90]. Compared to other genomic editing techniques, CRISPR-Cas9 is easier to perform, produces less off-target effects and is capable of multiple genome editing [91].

Genome editing could be applied in Reproductive Medicine to correct disease-causing mutations to avoid their inheritance by the offspring. Indeed, this approach has recently shown good results for the correction of a heterozygous mutation in the *MYBPC3* gene of human zygotes that causes hypertrophic cardiomyopathy [124]. Nevertheless, the reproducibility of this technique should further be revised before it is attempted in clinic, as there exist evidence of off-target effects and mosaic-embryos in previous studies [92,93].

Gene editing in gametes is another promising strategy for the correction of mutations that raise less ethical issues than manipulation of embryos. Recent reports have already achieved the correction of a mutation in the *Cryg* gene in mouse SSCs, thus obtaining progeny that are rid of eye-cataracts [94]. On top of that, the use of the CRISPR/Cas9 system in oocytes may also be a solution to avoid the transmission of mitochondrial DNA mutations to the offspring [95].

Likewise, CRISPR/Cas9 genomic edition in gametes from oncological patients subjected to fertility preservation may be an interesting approach to avoid the transmission
of genetic alterations causing cancer such as \textit{BRCA1}/\textit{BRCA2} mutated alleles causing breast and ovarian cancer. Thus, its application to gametes/gonadal tissue from oncological patients subjected to fertility preservation represents a promising challenge.
FIGURE AND TABLE LEGENDS

Figure 1. Schematic diagram of the fertility preservation options for female cancer patients.

Green arrows indicate techniques that are currently in use or have demonstrated their feasibility in clinical practice by healthy newborn deliveries. Red arrows indicate techniques that are considered experimental/at research stage. Embryo manipulation and IVM procedures susceptible of genetic edition are highlighted by DNA-scissors icon. Definitions: ART: Assisted Reproductive Techniques.
Figure 2. Schematic diagram of the fertility preservation options for male cancer patients.

Green arrows indicate techniques that are currently in use or have demonstrated their feasibility in clinical practice by healthy new born deliveries. Blue arrows indicate techniques that are currently under investigation. In vitro culture of isolated SSCs susceptible of genetic edition is highlighted by DNA-scissors icon. Definitions: ART: Assisted Reproductive Techniques; SSC: Spermatogonial Stem Cell; SSCT: Spermatogonial Stem Cell Transplantation.
investigated as strategies to restore fertility of cancer patients and others unable to produce mature gametes. In vitro propagation of iPSCs susceptible of genetic edition is highlighted by DNA-scissors icon. Definitions: ART: Assisted Reproductive Techniques; iPSC: induced Pluripotent Stem Cells; EpiLCs: Epiblast-like cells; PGCLCs: Primordial germ cell-like cells.
Table I: Factors affecting the reproductive outcome in cancer patients. Current and future preventive strategies.

<table>
<thead>
<tr>
<th>FACTOR</th>
<th>EFFECT</th>
<th>CLINICAL- BIOTECHNOLOGICAL STRATEGIES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUMOR</td>
<td>Invasion and direct damaging effect on:</td>
<td>Improve cancer screening methods (localized and reduced size of tumor at diagnosis)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Ovaries/Testes</td>
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<tr>
<td></td>
<td>• Uterus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Hypothalamic- pituitary axis (gonadotropin deficiency)</td>
<td></td>
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</tr>
<tr>
<td>SURGERY</td>
<td>Gonadal removal</td>
<td>Fertility-sparing surgery preserving gonads</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gonadal anatomical disruption: Obstruction of sperm transport</td>
<td>Minimally invasive surgical procedures- Robotic technology</td>
<td>[125]</td>
</tr>
<tr>
<td>GONADAL RADIOTHERAPY AND CHEMOTHERAPY</td>
<td>Damage to the seminiferous epithelium (spermatogonia and Sertoli cells) and Leydig cells</td>
<td>Shielding of the gonadal area</td>
<td></td>
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<tr>
<td></td>
<td>Depletion of primordial follicles - Impairment of follicular maturation</td>
<td></td>
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<tr>
<td></td>
<td>Uterine damage (volume, vascularization, endometrial thickness)</td>
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<tr>
<td></td>
<td>FP methods:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Oocyte vitrification</td>
<td></td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>• Embryo cryopreservation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Ovarian cortex cryopreservation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Sperm freezing</td>
<td></td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>• Cryopreservation of immature testicular tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adaptation of treatments to reduce potential reproductive toxicity</td>
<td></td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>High-precision modern radiation therapy techniques (eg intensity modulated radiation therapy, IMRT)</td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td>CRANIAL RADIOTHERAPY</td>
<td>Damaging effect on the hypothalamic- pituitary axis</td>
<td>High-precision modern radiation therapy techniques (eg intensity modulated radiation therapy, IMRT)</td>
<td>[127]</td>
</tr>
<tr>
<td>TYPE OF CANCER</td>
<td>Impaired semen quality has been associated with malignant diseases (eg. testicular cancer, Hodgkin’s lymphoma, leukemia or gastrointestinal malignancies)</td>
<td>Improve cancer screening methods</td>
<td>[128]</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>OTHER FACTORS</td>
<td>Clinical situation of the patient (eg. pain, pyrexia, anorexia) Psychological effects of cancer may affect future sexual relationships and therefore the reproductive outcome of patients</td>
<td>Improve clinical condition. Psychological counseling</td>
<td>[129]</td>
</tr>
</tbody>
</table>

**All factors above may be influenced/modulated by:**

- Age/sex
- Previous health status (fertility condition)
- Genetic background

[8, 130]
Table II: Estimated risk of infertility related to chemotherapy agents in pediatrics and adults, according to gender. ND: Not determined dose.

<table>
<thead>
<tr>
<th>RISK</th>
<th>MALES</th>
<th>FEMALES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High Risk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• &gt; 80% risk of prolonged azoospermia in males, or amenorrhea in females</td>
<td>Cyclophosphamide &gt;19g/m(^2)*</td>
<td>Cyclophosphamide</td>
<td>[5, 131, 132] [133]</td>
</tr>
<tr>
<td></td>
<td>Busulfan &gt;600mg/m(^2)</td>
<td>Busulfan &gt;600mg/m(^2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Melphalan &gt;140mg/m(^2)</td>
<td>Melphalan &gt;140mg/m(^2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Procarbazine 4g/m(^2)</td>
<td>Procarbazine ND*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ifosfamide &gt;52g/m(^2)</td>
<td>Ifosfamide ND**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cisplatin &gt;600mg/m(^2)</td>
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</tr>
<tr>
<td></td>
<td>Chlorambucil 1.4g/m(^2)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Carmustine 1g/m(^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lomustine 500mg/m(^2) **</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* 7.5-19g/m(^2) doses are of intermediate-high risk</td>
<td>* Recent studies suggest less impact in fertility even with high doses (11g/m(^2)) in pediatric population</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>** If treatment before puberty</td>
<td>** Ovarian function recovery has been described with doses of 60g/m(^2)</td>
<td>[135]</td>
</tr>
<tr>
<td><strong>Intermediate Risk</strong></td>
<td>Thiotepa 400mg/m(^2)</td>
<td>Thiotepa 400mg/m(^2)</td>
<td>[135] [5]</td>
</tr>
<tr>
<td>• 20-80% risk of prolonged azoospermia in males or amenorrhea in females</td>
<td>Doxorubicin &gt;770mg/m(^2)</td>
<td>Cisplatin ND</td>
<td>[131, 135]</td>
</tr>
<tr>
<td></td>
<td>Cisplatin 400-600mg/m(^2)</td>
<td>Cisplatin ND</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td>Carboplatin &gt;2g/m(^2)</td>
<td>Carboplatin ND</td>
<td>[131, 135]</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide 7,5-19g/m(^2)</td>
<td></td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td>Ifosfamide 42-52g/m(^2)</td>
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</tr>
<tr>
<td></td>
<td>Carmustine 1g/m(^2)</td>
<td></td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>Lomustine 500mg/m(^2)*</td>
<td></td>
<td>[5]</td>
</tr>
</tbody>
</table>
- Prolonged azoospermia or amenorrhea in combination with other agents or high doses

- Effects less established in humans with limit of upper dose not clear, but usually considered gonadotoxic agents

<table>
<thead>
<tr>
<th>Low Risk</th>
<th>Cytarabine ND</th>
<th>Bleomycin ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D ND</td>
<td>Vincristine ND</td>
<td>Vinblastine ND</td>
</tr>
<tr>
<td>Methotrexate ND</td>
<td>6-Mercaptopurine ND</td>
<td>5-Fluoracil ND</td>
</tr>
<tr>
<td>Etoposide ND</td>
<td>Thioguanine ND</td>
<td>Gemcitabine ND</td>
</tr>
</tbody>
</table>

- < 20% risk of prolonged azoospermia in males or amenorrhea in females

- Temporary reductions in sperm count by affecting differentiating spermatogonia

- Null or slight decrease of the ovarian reserve
Table III. Estimated risk on infertility related to radiotherapy in pediatric and adult males and females.

<table>
<thead>
<tr>
<th>RISK</th>
<th>COMMENTS</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High Risk</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Total-body irradiation as conditioning therapy in stem cell transplant</td>
<td>[5, 137]</td>
</tr>
<tr>
<td></td>
<td>• Testicular radiotherapy:</td>
<td>[57, 135]</td>
</tr>
<tr>
<td></td>
<td>&gt; 2.5Gy in adult men</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 6Gy in prepubertal boys</td>
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</tr>
<tr>
<td></td>
<td>• Pelvic or whole abdominal radiotherapy:</td>
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</tr>
<tr>
<td></td>
<td>≥ 6Gy in adult women</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 10Gy in postpubertal girls</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 15Gy in prepubertal girls</td>
<td></td>
</tr>
<tr>
<td><strong>Intermediate Risk</strong></td>
<td>The testis tissue, especially SSC, is very sensitive to irradiation. The effects are dose-dependent:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• &lt; 1Gy: prolonged azoospermia or oligozoospermia (reversible damage).</td>
<td></td>
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<tr>
<td></td>
<td>• &gt; 4Gy: prolonged and/or permanent infertility (irreversible damage).</td>
<td></td>
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<tr>
<td></td>
<td>• 30Gy damage to Leydig cells in adults. Dosage &gt;20 is associated with Leydig cell disfunction in boys.</td>
<td></td>
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<tr>
<td></td>
<td>• Craniospinal radiotherapy ≥ 25-30Gy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Testicular radiation 1-6 Gy from scattered abdominal or pelvic radiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Pelvic or whole abdominal radiotherapy:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-10 Gy in postpubertal girls</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-15 Gy in prepubertal girls</td>
<td></td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td>Human oocytes are very sensitive to radiation and their damage is dose and age-dependent:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 2-3Gy: oocyte degeneration, follicular atrophy and cell death.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Uterine damage worsens at younger age.</td>
<td></td>
</tr>
</tbody>
</table>