1. Title Page

Title: Combining fertility preservation procedures to spread the eggs across different baskets: a feasibility study

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2. Abstract

Study question

How can fertility preservation techniques be combined? What is the reproductive potential following combinations of ovarian stimulation, in vitro maturation (IVM) and ovarian tissue cryopreservation in female patients seeking fertility preservation (FP)?

Summary answer

In selected patients, combining different FP procedures is a feasible approach. Reproductive outcomes after FP in patients who return to attempt pregnancy are promising.

What is known already?

Fertility preservation is increasingly performed in fertility clinics but an algorithm to select the most suitable FP procedure according to patient characteristics and available timeframe is currently lacking. Vitrification of mature oocytes (OV) and ovarian tissue cryopreservation (OTC) are most commonly performed, although in some clinical scenarios a combination of procedures including IVM, to spread the sources of gametes, may be considered in order to enhance reproductive options for the future.

Study design, size and duration:
Retrospective, observational study in a university-based, tertiary fertility center involving all female patients who underwent urgent medical FP between January 2012 and December 2018. Descriptive analysis of various FP procedures, either stand-alone or combined, was performed, and reproductive outcomes of patients who attempted pregnancy in the follow-up period were recorded.

Participants/materials, setting, methods:
In total, 207 patients underwent medical fertility preservation. Patient-tailored strategies and procedures were selected after multidisciplinary discussion. When deemed feasible, fertility preservation procedures were combined to cryopreserve different types of reproductive tissue for future use. The main primary outcome measure was the number of mature oocytes. Live birth rates (LBR) were evaluated in patients who returned for reproductive treatment.

Main results and the role of chance:
Among patients seeking FP, 95/207 (46%) had breast cancer, 43/207 (21%) had haematological malignancies, and 31/207 (15%) had a gynaecological tumour. Mean age was 27 +/- 8.3 years. Eighty-five (41.1%) patients underwent controlled ovarian stimulation (COS), resulting in 10.8 +/- 7.1 metaphase II (MII) oocytes for vitrification. Eleven (5.3%) patients had multiple COS cycles. Transvaginal oocyte retrieval for IVM was performed in 17 (8.2%) patients, yielding 9.2 +/- 10.1 MII oocytes. Thirty-four (16.4%) patients underwent OTC combined with IVM of oocytes retrieved from ovarian tissue “ex vivo” (OTO-IVM), yielding 4.0 +/- 4.3 MII oocytes in addition to ovarian fragments. Seventeen (8.2%) patients had OTC combined with OTO-IVM and transvaginal retrieval of oocytes for IVM from the contralateral ovary, resulting in 13.5 +/- 9.7 MII oocytes. In 13 (6.3%) patients, OTC with OTO-IVM was followed by controlled stimulation of the contralateral ovary, yielding 11.3 +/- 6.6 MII oocytes in total.

During the timeframe of the study, 31/207 (15%) patients have returned to the fertility clinic with a desire for pregnancy. Of those, twelve (38.7%) patients had preserved ovarian function and underwent ART treatment with fresh oocytes, resulting in nine (75%) live births (LB). The remaining 19 (61.3%) patients requested warming of their cryopreserved material because of ovarian insufficiency. Of those, eight (42.1%) patients had a LB, of whom three after OTO-IVM. To date, 5/207 patients (2.4%) achieved an ongoing pregnancy or live birth after spontaneous conception.

Limitations, reasons for caution:
Our FP program is based on a patient-tailored approach rather than based on an efficiency-driven algorithm. The data presented are descriptive, which precludes firm conclusions.

Wider implications of the findings:
Combining different FP procedures is a feasible approach as an endeavor to enhance the reproductive fitness of patients undergoing gonadotoxic treatment. Preliminary live birth rates after FP are promising in patients who return for fertility treatment, whether or not they present with ovarian insufficiency. Further follow-up studies of patients who had FP are warranted.

Study funding/competing interest(s):
There was no funding and no competing interests.

Trial registration number:
Not applicable

3. Key words
Fertility preservation, oncofertility, ovarian tissue cryopreservation, oocyte vitrification, IVM

4. Introduction
Advances in anticancer treatment have substantially improved survival rates in young individuals with cancer. Of 35,035 patients who were diagnosed with cancer in Belgium, 4,970 (14%) were in the reproductive phase of their lives (Belgian Cancer Registry, 2016). Furthermore, the population contains an increasing proportion of survivors of childhood cancer. In view of the growing population of long-term cancer survivors (Phillips et al., 2015) and given that the reproductive potential of these patients may be affected by chemotherapy and pelvic radiotherapy (Rodriguez-Wallberg and Oktay, 2014), fertility preservation has gained widespread acceptance (Martinez, 2017). Studies have shown that FP counseling with the option to preserve own gametes for deferred reproduction, may increase quality of life (Letourneau et al., 2012). FP counselling is nowadays considered as good clinical practice. Nevertheless, according to the results of a recent survey among 432 breast cancer survivors in the US, 67% of women of reproductive age who were diagnosed with breast cancer had not been counselled regarding the potential impact of cancer treatment on fertility. Consequently, they did not use FP strategies (Bressler et al., 2019). Lack of knowledge among patients and their treating physicians about the current status of FP procedures may be a major impediment to a more widespread use for FP referral.
Established fertility preservation methods include oocyte and embryo cryopreservation following controlled ovarian stimulation. Nonetheless, increasing evidence of restoration of ovarian function and spontaneous conception following orthotopic transplantation of ovarian tissue supports ovarian tissue cryopreservation as an established FP option for those patients who do not have time or are reluctant to undergo ovarian stimulation (De Vos et al., 2014; Jadoul et al., 2017; Donnez et al., 2017; Martinez F. 2017; Practice Committee of the American Society for Reproductive Medicine, 2019). Immature oocytes, obtained transvaginally or retrieved from ovarian tissue “ex vivo” can mature in vitro and may be an additional source of gametes for cryopreservation (Segers et al., 2015; Hourvitz et al., 2015). In view of the limited number of births after IVM of oocytes in cancer patients, the efficacy of this experimental technology has yet to be established (Son et al., 2019).

Evidence of reproductive outcomes in cancer survivors who underwent FP is scarce (Cobo et al., 2018; Díaz-Garcia et al., 2018), and an algorithm to select the most efficient FP procedures according to patient characteristics, tumour type and available timeframe is currently not available. A combination of multiple FP procedures in order to increase the sources of gametes with the intention to enhance the reproductive options of cancer survivors may be considered in selected patients. We here describe our experience with different FP procedures, either as a stand-alone intervention or combined and we present the reproductive outcomes of patients who attempted pregnancy in their follow-up period.

5. Materials and methods

Study design

All patients who were referred to our university-based, tertiary fertility center between January 2012 and December 2018 and who underwent fertility preservation for medical reasons were included. The majority of patients had a cancer diagnosis, although patients who required gonadotoxic treatment for non-malignant conditions were also included. Patients who underwent FP because of a family history of premature ovarian failure or because of non-medical reasons (i.e. planned oocyte freezing) were not
included in this study. All patients or their legal representatives (in case of minors) gave written in-
formed consent. Information was retrieved retrospectively from electronic medical records. Approval
for the analysis of retrospective data was given by the local Ethical Committee BUN 143201731279.

Interventions for FP

In general, controlled ovarian stimulation (COS) for oocyte and/or embryo vitrification was conducted
using random start recombinant follitropin alfa (rFSH), corifollitropin alfa or highly purified hMG
(HP-hMG) in a GnRH antagonist protocol (Cakmak et al., 2013). The gonadotropin starting dose was
selected at the physician’s discretion. Of note, 90% (n = 41/46) of all women diagnosed with hormone
positive breast cancer had 5mg daily letrozole cotreatment during gonadotropin stimulation. Final oo-
cyte maturation was induced by injection of 0.2mg triptorelin (Decapeptyl, Ipsen Pharma, Merelbeke,
Belgium; or Gonapeptyl, Ferring Pharmaceuticals, Aalst, Belgium) and/or by injection of 5000 IU of
hCG (Pregnyl, MSD, Oss, The Netherlands), as soon as the mean diameter of two to three leading fol-
licles was > 18 mm in size as observed on ultrasound scan (Cobo et al., 2016). Oocyte retrieval fol-
lowing COS was carried out 36h after ovulation trigger. Oocyte and embryo vitrification were per-
formed using the Vitrification kit media (Irvine Scientific, Tilburg, Netherlands) and high-security
straws (VHS Kit, CryoBiosystem, L’Aigle, France), as previously described (De Munck et al., 2015;
De Vos et al., 2016).

In selected cancer patients with an AFC of at least ten antral follicles per ovary, who declined COS or
in whom COS was not indicated because of lack of time, transvaginal antral follicle aspiration was
performed for the retrieval of immature cumulus-oocyte complexes (COC), followed by incubation of
these complexes in IVM media for 30 hours as previously described (Mostinckx et al., 2019). In these
cases, no ovulatory trigger was given before oocyte retrieval, although some patients received HP-
hMG pretreatment.

In prepubertal patients and in postpubertal cancer patients in whom urgent gonadotoxic therapy was
required and COS was not indicated, complete or partial unilateral oophorectomy was performed by
laparoscopy for ovarian tissue cryopreservation (OTCP). Ovarian tissue was transported in a sterile
0.9% saline solution on ice to the IVF laboratory within a time frame ranging from 10 min to a maximum of three hours in cases who underwent oophorectomy at another hospital. After processing the ovarian tissue for cryopreservation, the collection dish was examined for the presence of small COC that had been released from follicles that ruptured during the cutting process, as previously described (Segers et al., 2015). Ovarian cortex tissue strips of approximately 8x5mm were frozen using a slow freezing protocol with DMSO as cryoprotectant (adapted from Donnez et al., 2004). Where deemed feasible, in selected patients and after discussion with the referring oncologist and informed consent, the aforementioned interventions were combined.

Fertility treatment in patients who returned

All cancer survivors who returned to the clinic with a desire for pregnancy had ovarian reserve testing (serum levels of Antimüllerian hormone (AMH), FSH and estradiol and antral follicle count (AFC)) at their return visit. The AMH analysis method has been previously prescribed in detail (De Vos et al., 2018). According to these results and to patient preference, one of the following options was suggested: when ovarian reserve parameters were still compatible with an attempt to pursue pregnancy without using cryopreserved material, then patients embarked on either (i) follicle tracking and timed intercourse, (ii) controlled ovarian stimulation followed by IVF/ICSI, or (iii) a managed natural cycle followed by ICSI. Alternatively, if ovarian reserve parameters indicated severe follicle depletion or a postmenopausal status, patients went on to use their cryopreserved gametes, embryos or ovarian tissue. In both groups, when a frozen embryo transfer was performed, artificial supplemented cycles with exogenous estrogen and progesterone were used to prepare the endometrium (De Vos et al., 2016).

Primary outcome

The primary outcome is the number of mature oocytes obtained using the different FP interventions. Live birth rates (LBR) were evaluated in patients who returned for reproductive treatment.

Statistical analysis

Continuous data are presented as mean ± SD and categorical data are described by number of cases, including numerator and denominator, and percentages. Categorical data and continuous data that did
not show a normal distribution were analyzed by Pearson’s x2 test/Fisher exact test or Kruskal–Wallis, as appropriate.

6. Results

Indications for FP procedures

Among 207 patients who were referred to our centre for medical (oncological and non-oncological) FP, breast cancer (95/207, 46%) was the most common diagnosis, followed by haematological (43/207, 21%) and gynaecological tumours (31/207, 15%) (Figure 1). In breast cancer patients, 18/95 (18.9%) women were carrier of a BRCA 1/2 mutation. Other indications for FP included neurological cancers (18/207, 9%), sarcomas (8/207, 3%), colorectal cancer (6/207, 3%), nasopharyngeal cancer (1/207, 0.5%), thyroid cancer (1/207, 0.5%) and pancreas cancer (1/207, 0.5%). Three patients (3/207, 1%) underwent FP in view of potential gonadotoxic treatment of a non-malignant condition (uterine arteriovenous malformation, myelofibrosis and Bare lymphocyte syndrome). None of the patients had FP in view of pelvic surgery for endometriosis. Sixteen (7.7%) patients had previously been treated with chemotherapy before they had FP. Among those, ten patients were post-pubertal and six were at a pre-pubertal stage. The majority (7/10) of the post-pubertal patients had FP at least six months after chemotherapy. The remaining three patients had FP within six months after the end of chemotherapy; two of those underwent OTC (at one and three months after chemotherapy, resp.), one patient had COS followed by oocyte vitrification five months after chemotherapy. Four out of six pre-pubertal patients who had OTC following chemotherapy did so within one and five months after chemotherapy. Two further patients underwent OTC + IVM of oocytes retrieved “ex vivo” (Ovarian Tissue Oocytes, OTO-IVM) after two resp. six months after chemotherapy. Women who underwent FP because of a family history of premature ovarian failure or who had planned oocyte cryopreservation to anticipate age-related infertility were not included in this study.

FP procedures, stand-alone or combined
Figure 2 portrays the distribution of FP procedures that were applied during the study period, either as stand-alone procedures or combined. Details about these FP procedures are described in the following paragraphs.

Characteristics of patients who underwent various types of FP procedures are presented in Table 1. Post-pubertal and pre-pubertal patients were considered separately. The characteristics of postpubertal patients included age, BMI, circulating AMH and antral follicle count at FP intake. Age and BMI are presented for the prepubertal patients. A Kruskal-Wallis test was performed comparing the different groups.

Controlled ovarian stimulation was performed in 85/207 (41.1%) patients. Eleven (5.3%) patients underwent two or more rounds of COS (denoted as consecutive cycles of COS). In total, 116 COS cycles were performed. In the vast majority of patients, ovarian stimulation was performed using recombinant FSH in a GnRH antagonist protocol; a long GnRH agonist protocol was prescribed in three (2.6%) cycles. An early follicular phase endocrine profile at the start of COS was noted in 78/116 (67.2%) cycles, 24/116 (20.7%) cycles being luteal phase stimulated cycles. The endocrine profile at the start of COS was unknown in 14 cycles. Cycles of COS in a GnRH antagonist protocol had a mean duration of 9.3 +/- 1.4 days of stimulation, as compared to 23.3 +/- 1.5 days in the small subset of long GnRH agonist cycles. The final maturation was initiated with an agonist trigger in 102/113 (90.3%) GnRH antagonist cycles; in 9/113 (8%) GnRH antagonist cycles a hCG trigger was administered. A dual trigger was used in two GnRH antagonist cycles. Table 2 presents the general characteristics of controlled ovarian stimulation (COS) cycles in patients diagnosed with breast (n = 56), haematological (n = 10) or gynaecological cancer (n = 18), which represent the three most frequent indications for FP.

Transvaginal oocyte retrieval for IVM was performed in 17/207 (8.2%) patients. Three patients were pretreated with gonadotropins. The transvaginal IVM retrieval was performed in the early follicular phase in 14/17 (82.4%) patients and in the luteal phase in 3/17 (17.6%) women.

When OTC was performed, this procedure was combined with OTO-IVM in the majority of patients. In total, 34/207 (16.4%) patients (nine pre-pubertal and 25 post-pubertal cases) had OTC combined
with OTO-IVM, whereas 10 patients (of which four were prepubertal children) underwent OTC only.

Of these 34 patients, two underwent oophoropexy during the laparoscopy for OTC.

The combination of transvaginal oocyte retrieval for IVM followed by OTC of the contralateral ovary and OTO-IVM was performed in 17/207 (8.2%) patients. In general, the ovary with the highest AFC was selected for transvaginal oocyte retrieval.

13/207 (6.3%) patients had a combination of OTC with OTO-IVM and started controlled stimulation of the contralateral ovary on the same day, after the laparoscopy. Among these, nine had an early follicular phase basal endocrine profile, whereas three patients were in the luteal phase at the start of the FP procedure.

Other combinations of FP procedures included controlled ovarian stimulation followed by transvaginal oocyte retrieval for IVM as soon as the endocrine profile had returned to basal values (6/207 patients).

In 7/207 patients we performed this combined strategy in the reverse order, i.e. COS was initiated after transvaginal oocyte retrieval for IVM. Six further patients had OTC with OTO-IVM after one cycle of COS for oocyte vitrification; however, because COS resulted in extensive damage to the ovarian cortex tissue upon cryopreservation, this strategy has been abandoned. In one patient with a borderline ovarian mucinous cystadenoma who underwent oophorectomy, ovarian cortex cryopreservation was not feasible because of the possibility of tumour invasion. In that patient, OTO-IVM was performed, unfortunately not resulting in any metaphase II oocytes. However, that same patient got pregnant at a later stage after ovarian stimulation of the contralateral ovary and FET. Ten patients, of whom four were prepubertal, underwent OTC without OTO-IVM, because no COC were identified during ovarian cortex processing in the lab in these patients.

**Metaphase II oocyte yield**

Table 3 shows the average mature metaphase II oocyte yield after various stand-alone or combined FP procedures. Patients who underwent one cycle of COS yielded 10.8 +/- 7.1 MII oocytes for vitrification. When consecutive cycles of COS were performed, 18.2 +/- 12.7 mature oocytes were retrieved.

In six cases COS was performed twice. Three further patients had three cycles of COS and two further
women had four cycles of COS (prior to ovarian surgery because of teratomas). Performing COS in
the early follicular phase yielded on average 10.6 +/- 6.4 MII oocytes, whereas luteal phase stimula-
tion resulted in a mean of 11.7 +/- 9.1 mature oocytes (p-value: 0.5).

A transvaginal oocyte retrieval of non-stimulated ovaries resulted in 9.2 +/- 10.1 MII oocytes after
IVM. In this group of patients, the oocyte maturation rate was 49.3%. Three of these patients under-
went transvaginal oocyte retrieval for IVM in the luteal phase. This resulted in 3.3 +/- 2.5 MII oocytes
on average. Performing this procedure in the early follicular phase in 14 patients yielded 10.1 +/- 11.2
mature oocytes per patient. The maturation rate was similar in both groups (49.2 % vs 55.0 %, resp.,
p-value: 0.8).

Women who had a combination of OTC and OTO-IVM achieved a yield of 4.1 +/- 3.7 metaphase oo-
cytes, with a mean oocyte maturation rate of 34.8% after IVM in the post-pubertal phase. In these
cases, a follicular density in the cryopreserved ovarian tissue of 15.4 +/- 21.2 primordial follicles/mm²
was noted. For the pre-pubertal patients, a mean yield of 3.7 +/- 5.7 metaphase II oocytes was
achieved and a follicular density of 40.8 +/- 37.6 primordial follicles/mm² was observed.

In the group of post-pubertal patients who had transvaginal oocyte retrieval for IVM followed by OTC
and OTO-IVM, the average yield was 13.5 +/- 9.7 MII oocytes. More specifically, a mean of 3.9 +/-
4.2 MII oocytes were derived from OPU IVM and 9.5 +/- 6.9 metaphase II oocytes were collected
during OTO-IVM (p-value: 0.007). In these patients, the oocyte maturation rate was 37.5 +/- 24.6 %
for oocytes originating from OPU-IVM and 39.7 +/- 19.3 % for OTO-IVM oocytes (p-value: 0.8).

The follicular density of the cryopreserved ovarian tissue in this patient subset was 33.9 +/- 53 per
mm².

The combination of OTC (unilateral oophorectomy) and OTO-IVM followed by COS of the contrala-
teral ovary yielded a total number of 11.3 +/- 6.6 mature oocytes on average. On average, 4.6 +/- 2.6
mature oocytes for vitrification were obtained after COS. We noted 6.7 +/- 6.1 metaphase II oocytes
after OTO-IVM (p-value: 0.26). The maturation rate was 74.3 +/- 14.3 % for oocytes derived from
COS and 41.4 +/- 22.7 % after OTO-IVM (p-value< 0.001). A follicular density of 13.1 +/- 13.2 primordial follicles per mm² was noted.

Other combinations of FP procedures, as detailed above, yielded on average 9.1 +/- 7.5 MII oocytes in 26 post-pubertal patients. The mean follicular density as observed in the ovarian tissue was 60.2 +/- 79.4 per mm².

A secondary analysis of metaphase II oocyte yield after COS was performed across subgroups of patients with either breast cancer, haematological cancer or gynaecological cancer. The metaphase II oocyte yield was comparable in these subgroups (table 3c).

Follow-up and live birth rate

Among the 207 patients who were included in this retrospective analysis, 31 (15%) women returned to the clinic with a desire for pregnancy during the timeframe of the study, after a mean elapsed time of 29.4 +/- 13.8 months since the intervention for FP. Written permission was obtained from the oncologist in all patients who returned. In twelve of these (38.7%), the menstrual pattern and/or endocrine profile were compatible with a premenopausal status, which made them eligible for fertility treatment without using their cryopreserved material. The mean serum AMH in these patients was 1.1 +/- 1.3 ng/mL. Eleven out of twelve underwent ART (COS or natural cycle IVF/ICSI depending on ovarian reserve parameters), one patient had ovulation induction.

In this group of twelve cancer survivors with preserved ovarian function, nine live births (75%) were obtained after fertility treatment without utilisation of cryopreserved material. Of those, five were achieved after COS and ICSI, of which three had occurred after PGT-M. One patient had a live birth after managed natural cycle and ICSI. Two further live births were obtained after vitrified-warmed embryo transfer following ICSI in a managed natural cycle and elective vitrification of embryos. Finally, one patient had a live birth after ovulation induction and timed intercourse.

The remaining 19 patients (61.3%) had amenorrhoea or severe oligomenorrhoea with hormone profiles that were deemed unsuitable for fertility treatment with fresh oocytes. In 17/19 patients, oocytes or embryos that had been vitrified before chemotherapy were warmed. This approach resulted in a live
birth in $8/17$ patients (47.1%). In five of these, a live birth resulted from FET of embryos that had been
vitriﬁed after ovarian stimulation prior to chemotherapy. Among these ﬁve patients, there was one pa-
tient in whom PGT-M for BRCA1 had been performed prior to embryo vitriﬁcation. Another patient
had COS as FP procedure prior to chemotherapy followed by oocyte warming, ICSI and embryo trans-
fer in an artiﬁcial cycle. This resulted in a live birth. In two further patients live births were obtained
after vitriﬁed-warmed transfer of embryos derived from OTO-IVM. In one patient oocyte warming
after OTO-IVM was performed, followed by ICSI and embryo transfer, resulting in a live birth.

Two out of 19 patients underwent ovarian tissue transplantation after utilization of all cryopreserved
embryos or oocytes. No pregnancy has been achieved in these two patients during the study timeframe
(unti December 2018).

A spontaneous pregnancy was noted in ﬁve patients. These resulted in three live births and two ongo-
ing pregnancies. Figure 3 presents a graphical overview of the trajectory in patients who returned for
fertility treatment. Figure 4 depicts the various combinations of FP procedures that were applied, with
respect to the available timeframes.

7. Discussion

Discussing FP in young cancer patients is essential and is becoming a key component of routine onco-
logical health care. Different guidelines have been established with recommendations regarding possi-
ble interventions for fertility preservation depending on patient age, tumour type and available
timeframe. These guidelines, of which ESMO 2013 (Peccatori et al., 2014), ASRM/ESHRE 2017
(Martinez, 2017) and ASCO 2018 (Oktay et al., 2018) are commonly cited, suggest that ovarian stimu-
lation and embryo or oocyte cryopreservation should be discussed as the ﬁrst-line FP method. Ovarian
tissue cryopreservation is an alternative option, and no longer considered as an experimental procedure
according to the recent ASRM/ESHRE guidelines (Martinez, 2017; Practice Committee of the Ameri-
can Society, 2019). Even though in the majority of cases the selection of the type of FP intervention is
based on age, pubertal status, tumour type, experience of the fertility center and patient choice, this
selection is not always straightforward. Indeed, in the absence of an efficiency-based algorithm, controlled ovarian stimulation followed by oocyte vitrification is the strategy of choice in the majority of postpubertal patients whose condition does not require an immediate start of chemotherapy. Oocyte vitrification has been performed efficiently in fertility clinics for over a decade (Kuwayama et al., 2005), both in the setting of egg donation as for the indication of non-medical and medical fertility preservation. The use of oocyte vitrification for oncofertility has been fueled by the publication of favourable outcomes with vitrified oocytes in egg donation programs (Cobo et al., 2010), and by evidence suggesting that ovarian stimulation for oocyte cryopreservation in patients with hormone-dependent malignancies is safe (Kim et al., 2016; Rodgers et al., 2017). Moreover, oocyte cryopreservation as opposed to ovarian tissue cryopreservation does not require a surgical procedure which may be an important element in the decision-making process of the cancer patient. Nevertheless, the utilization rate of vitrified oocytes in cancer survivors is less than 10% and reports of clinical outcomes of IVF using vitrified-warmed oocytes in this population are still scarce (Cobo et al., 2018; Diaz-Garcia et al., 2018). Furthermore, outcome data have only been published by a small number of expert centers and these results cannot be readily extrapolated to centers with less experience. Indeed, centerspecific information with regard to clinical outcomes after oocyte vitrification for fertility preservation is not available. On a similar note, outcome data following cryopreservation of ovarian cortex are also limited, although it appears from data published by centers with sufficient expertise that ovarian function after grafting is restored in more than 90% of patients and that approximately half of the reported pregnancies are spontaneous pregnancies (Diaz-Garcia et al., 2018). Therefore, in view of the dependence on sufficient expertise of fertility preservation procedures and in view the inherent risk of failure to achieve a pregnancy if one single source of frozen material is available, the combination of oocyte vitrification and ovarian cortex freezing may reduce the failure rate by spreading the risk. Moreover, there is preliminary data to suggest that cryopreservation of ovarian tissue, followed immediately by ovarian stimulation does not impair the number of oocytes retrieved (Dolmans et al., 2014). Therefore, ovarian tissue grafting as an additional option in cancer survivors who fail to become pregnant after using their vitrified oocytes, may have the potential to enhance the chance of a live birth in these patients. Although the data from the study presented here do not allow us to confirm the hypothesis that
live birth rates in selected cancer patients may be increased if multiple fertility preservation procedures
are combined, compared to performing one procedure only, they illustrate the feasibility of combining
multiple procedures in a substantial group of cancer patients. Moreover, the number of mature oocytes
available for cryopreservation did not appear to be impaired by such a strategy according to our re-
sults. Indeed, when comparing the number of mature oocytes following COS, which was the FP proce-
dure most frequently performed, with combinations of OPU IVM + OTC + OTO-IVM or OTC +
OTO-IVM + COS, similar oocyte yields were observed.

On the other hand, the aim of this study was not to identify the best possible approach in cancer pa-
tients seeking fertility preservation. At best, the approach has to be patient-tailored, considering the
tumour type, the available time for FP before the start of the gonadotoxic treatment, the overall condi-
tion of patient and the patient’s own preference after detailed counselling. Based on our experience,
the combination of multiple interventions for FP within a limited timeframe requires multidisciplinary
care coordination, to ensure that the oncologist, gynecologist and reproduction endocrinologist work
together efficiently, to certify that counseling can be delivered quickly, and that appropriate options
are put into place without delay.

The mean number of mature oocytes after controlled ovarian stimulation in our population was 10.8
+/- 7.1, which is slightly higher than the oocyte yield reported by Decanter et al (6.2 +/- 4.7) (De-
canter et al., 2018) and by Diaz-Garcia et al (8.1 +/- 6.6) (Diaz-Garcia et al., 2018), in spite of compa-
rable age, ovarian reserve markers and cycle characteristics across these studies. It has been suggested
that cancer may exert a negative impact on oocyte quality and/or oocyte maturation (Paradisi et al.,
2016), although the choice of the starting dose of gonadotropins and/or the choice of ovulation trigger
may also modulate the yield of mature oocytes. Indeed, the use of a GnRH agonist trigger instead of a
hCG trigger may result in an increased number of metaphase II oocytes (Cakmak et al., 2013). Alt-
ough Cobo et al reported a linear correlation between the number of vitrified-warmed oocytes availa-
ble for ICSI and the live birth rate, this correlation does not imply that for a given cancer patient un-
dergoing COS for FP, increasing the gonadotropin dose to intensify ovarian stimulation and to obtain
a higher number of oocytes should per se result in a higher live birth rate. Based on the data of Cobo et
al., suggesting that 10 metaphase II oocytes in cancer survivors corresponds to a LBR of 40% on average, and those of Diaz-Garcia et al showing that roughly two thirds of cancer survivors who return to use their vitrified oocytes, fail to become pregnant with their vitrified oocytes, it is clear that the majority of cancer survivors will have to turn to other methods to fulfill their desire to have a child after chemotherapy. In view of this, combining COS with OTC could potentially enhance the reproductive options, at least for a subset of selected cancer patients, and in centers with sufficient expertise.

Nevertheless, according to our data, the mean number of metaphase II oocytes after a combination of FP procedures was comparable to the mature oocyte yield after one round of controlled ovarian stimulation. Combining different FP techniques may not increase the oocyte yield in a cancer patient, but this approach may spread out the risk of failure upon return to clinic, when the patient is in remission after cancer and when she has a desire for pregnancy. Moreover, an approach tailored to the patient’s preference and with the aim to optimally exploit the available sources of reproductive material could have a favourable impact on the patient’s decisional conflict with regard to FP. Finally, when the timeframe for FP is limited, combining procedures that can be performed at short notice, such as OPU IVM and OTC + OTO-IVM can be highly relevant. Nevertheless, the potential added value of combining FP procedures has to be considered in the context of availability of resources, and combining FP procedures will more realistic for women whose treatment is reimbursed by their healthcare insurance.

The use of IVM in the setting of fertility preservation requires further scrutiny. Although it may seem from our data that the mature oocyte yield following a combination of ovarian stimulation and ovarian tissue cryopreservation with IVM of oocytes derived from extracorporeal ovarian tissue is comparable to the yield of ovarian stimulation only, it is clear from the literature that the potential of these OTO-IVM oocytes to result in a live birth may be significantly lower compared to the potential of mature oocytes retrieved after ovarian stimulation (Son et al., 2019). We noted that the technique of OTO-IVM of COC derived from OTC resulted on average in between 4.1 and 9.5 mature oocytes, which compares favourably with the metaphase II oocyte yield after COS and OPU IVM. In our study, the majority of patients who had OTC underwent removal of a whole ovary, whereas only a small subset
of patients had a partial oophorectomy. The difference in maturation rate was not statistically different comparing the two sources of immature oocytes (37.5 +/- 24.6 % for OTO-IVM and 39.7 +/- 19.3 % for OPU IVM). Of note, the maturation rate of oocytes derived from COS was substantially higher (74.3 +/- 14.3 %). Further research is needed to optimize the composition of culture media adapted to the requirements of immature COC from small antral follicles (Sanchez et al, 2019). The analysis of primordial/primary follicle density in the ovarian cortex of our patient cohort reveals substantial heterogeneity. Although an association has been shown between primordial follicle density and serum AMH levels in young cancer patients (Sermondade et al., 2019), the value of this parameter in a single sample is questionable. In pre-pubertal patients one of the only options for FP treatment is OTC. The possibility of combining this procedure with OTO-IVM does not cause a delay for the patient, although the true potential of OTO-IVM oocytes in young children is yet to be established. Indeed, follicle developmental competence evolves through childhood and adolescence and a high proportion of abnormal non-growing follicles have been observed in prepubertal ovaries (Anderson et al., 2014).

Since none of our patients who returned to the clinic with a desire for pregnancy had FP before puberty, we do not have any data about the fertility potential of prepubertal oocytes or ovarian tissue.

When comparing the different groups, statistically significant differences were noted with regards to age, AMH, AFC and number of metaphase II oocytes. On one hand this can be explained by the patient-tailored method of choosing the preferential FP technique. In post-pubertal women, the assessment of the fertility status including AMH and AFC prior to gonadotoxic therapy is important to evaluate the feasibility of OPU IVM and/or OTO-IVM. In our series, the markers of ovarian reserve were indeed higher in patients who underwent OPU IVM as a stand-alone FP treatment or combined with OTC + OTO-IVM. However, we noted a slightly lower number of MII oocytes per AMH-AFC-threshold in our study compared to the article of Sonigo et al, who showed that an AFC of 28, 20 and 19 (with corresponding serum AMH concentrations of 3.9, 3.7 and 3.5 ng/mL, respectively) were required in order to obtaining at least 15, 10 or 8 frozen IVM oocytes. In our study, we observed a mean AFC of 35.8 +/- 29.4 and AMH of 5.4 +/-7.6 in patients undergoing OPU IVM as a FP procedure,
yielding a mean number of 9.2 +/- 10.1 mature oocytes. In the subgroup of patients who had a combination of OPU IVM + OTC + OTO-IVM, yielding on average 13.5 +/- 9.7 MII oocytes, the mean serum AMH concentration was 5.7 +/- 6.0 ng/ml and the mean AFC was 34.3 +/- 22.0. Nevertheless, the small patient numbers of our subgroups have to be considered when interpreting these results.

Because the timeframe of FP is important for these patients, FP has to be scheduled as urgently as possible. It has been shown previously that commencing ovarian stimulation or performing a OPU IVM either in the follicular phase or in the luteal phase does not have an impact on the final MII oocyte yield (Cakmak et al., 2013; Grynberg et al., 2016). In our study, we did not observe any difference in oocyte yield between follicular and luteal phase stimulation either. Hence, we suggest that a random-start stimulation can be considered effective and time saving.

When comparing different indications for FP, no difference in oocyte yield was observed. Nevertheless, the tumour type and the urgency of starting gonadotoxic treatment play an important role in the decision-making process concerning the FP procedure. Hematological cancers are more often diagnosed in younger and/or pre-pubertal patients where chemotherapy cannot be postponed and sufficient time for ovarian stimulation is often not available.

We are aware that this is a descriptive study with a limited number of patients and significant patient heterogeneity. Moreover, our selection of FP methods and possible combinations evolved during time. Therefore, our data preclude firm conclusions and need confirmation in larger studies including follow-up of cancer survivors who return to use their cryopreserved material.

Finally, the observed LBR in the small proportion of patients (15%) who have so far returned to our clinic with a desire of pregnancy after gonadotoxic treatment is promising. The return rate of our sample is in line with figures reported in previous studies, varying from 4.8% and 6.2% in patients who underwent oocyte vitrification and OTC, respectively (Diaz-Garcia et al., 2018), to 7.4% (Cobo et al., 2018) and 29% overall (Rodriguez-Wallberg et al., 2019). We observed that nearly half of our patients
who used cryopreserved material became pregnant. This is in line with previous studies reporting LBR after oocyte vitrification, ranging from 32.6% (Diaz-Garcia et al., 2018) to 42.1% (Cobo et al., 2018). Of note, three of our patients achieved a live birth using cryopreserved material following a combination of FP procedures. Moreover, the observed clinical outcome is also promising in those patients in whom the ovarian function after cancer treatment was still sufficient to allow ART with fresh oocytes. In our study, only two patients so far underwent ovarian tissue transplantation after utilizing all of their cryopreserved embryos or oocytes. A longer follow-up is needed to assess use and live birth rate of ovarian tissue transplantation in our centre.

8. Author's roles

Sien Delattre: concept and draft of the article
Ingrid Segers: interpretation and editing of the article
Ellen Van Moer: interpretation and editing of the article
Panagiotis Drakopoulos: interpretation and editing of the article
Ileana Mateizel: interpretation and editing of the article
Lissa Engels: interpretation and editing of the article
Herman Tournaye: interpretation and editing of the article
Michel De Vos: concept and final revision of the article

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10. Funding

Not applicable.

11. Conflict of interest

None to declare.

12. Reference list


Figure 1. Indications for fertility preservation (FP) treatment (total n = 207 patients).

Figure 2. Distribution of FP procedures (total n = 207 patients). COS, controlled ovarian stimulation; OPU-IVM, transvaginal oocyte retrieval followed by IVM of oocytes; OTC, ovarian tissue cryopreservation; OTO-IVM, IVM of oocytes retrieved from ovarian tissue “ex vivo”.
Figure 3. Distribution of diagnoses within the different FP treatment groups.

Figure 4. Selection of FP procedures. AFC, antral follicle count; AMH, anti-Müllerian hormone.
Figure 5. Live births in patients who returned. 

1ICSI, COS followed by ICSI and fresh ET; 2ICSI PGT-M, COS followed by ICSI, pre-implantation genetic testing for monogenic disorders and ET; 3MNC, managed natural cycle IVF/ICSI; 4Ov and TI, ovulation induction and timed intercourse; 5FET after COS, frozen embryo transfer following COS as FP method; 6FET after OTO-IVM, frozen embryo transfer following ex vivo IVM as FP method; 7WOET, frozen transfer of an embryo generated using ICSI of a warmed oocyte following OTO-IVM as FP method.

### Table I. General characteristics: post-pubertal patients.

<table>
<thead>
<tr>
<th>FP treatment</th>
<th>All (n = 194)</th>
<th>COS (n = 85)</th>
<th>OPU-IVM (n = 17)</th>
<th>OTC + OTO-IVM (±euphorophy) (n = 25)</th>
<th>OTC + COS (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>28.9 ± 6.1</td>
<td>30.4 ± 5.8</td>
<td>27.8 ± 6.7</td>
<td>27.9 ± 6.6</td>
<td>25.9 ± 4.8</td>
</tr>
<tr>
<td>BMI (kg/m²), mean ± SD</td>
<td>22.9 ± 4.9</td>
<td>22.7 ± 4.0</td>
<td>23.8 ± 5.4</td>
<td>22.0 ± 4.2</td>
<td>24.8 ± 7.9</td>
</tr>
<tr>
<td>AMH (ng/ml), mean ± SD</td>
<td>3.1 ± 3.8</td>
<td>2.6 ± 2.1</td>
<td>5.4 ± 7.6</td>
<td>1.7 ± 1.0</td>
<td>5.7 ± 6.0</td>
</tr>
<tr>
<td>AFC, mean ± SD</td>
<td>19.7 ± 16.9</td>
<td>16.0 ± 10.2</td>
<td>35.8 ± 29.4</td>
<td>11.1 ± 5.6</td>
<td>34.3 ± 22.0</td>
</tr>
</tbody>
</table>

AFC: antral follicle count; AMH: anti-Müllerian hormone; COS: controlled ovarian stimulation; OPU-IVM, transvaginal oocyte retrieval followed by IVM of oocytes; OTC, ovarian tissue cryopreservation; OTO-IVM, IVM of oocytes retrieved from ovarian tissue. *ex vivo*.

### Table II. General characteristics: pre-pubertal patients.

<table>
<thead>
<tr>
<th>FP treatment</th>
<th>All (n = 13)</th>
<th>OTC (n = 4)</th>
<th>OTC + OTO-IVM (n = 9)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>5.2 ± 4.6</td>
<td>5.5 ± 7.1</td>
<td>5.1 ± 3.6</td>
<td>0.89</td>
</tr>
<tr>
<td>BMI (kg/m²), mean ± SD</td>
<td>16.1 ± 2.2</td>
<td>16.2 ± 1.9</td>
<td>16.0 ± 2.6</td>
<td>0.89</td>
</tr>
</tbody>
</table>
### Table III
General characteristics of ovarian stimulation cycles in patients with breast, haematological and gynaecological cancer.

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer (n = 56)</th>
<th>Haematological cancer (n = 10)</th>
<th>Gynaecological cancer (n = 18)</th>
<th>P-value</th>
<th>Total COS cycles (n = 116)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early follicular phase start, n (%)</td>
<td>42 (75)</td>
<td>7 (70)</td>
<td>16 (89)</td>
<td>N/A</td>
<td>78 (67.2)</td>
</tr>
<tr>
<td>Luteal phase start, n (%)</td>
<td>14 (25)</td>
<td>3 (30)</td>
<td>2 (11)</td>
<td>N/A</td>
<td>24 (20.7)</td>
</tr>
<tr>
<td>GnRH antagonist protocol, n (%)</td>
<td>56 (100)</td>
<td>10 (100)</td>
<td>18 (100)</td>
<td>N/A</td>
<td>111 (97.4)</td>
</tr>
<tr>
<td>Long GnRH agonist protocol, n (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>3 (2.6)</td>
</tr>
<tr>
<td>rFSH vs. menotropins, n (%)</td>
<td>53 (95) vs 3 (5)</td>
<td>8 (89) vs 1 (11)</td>
<td>17 (94) vs 1 (6)</td>
<td>N/A</td>
<td>102 (87.9) vs 14 (12.1)</td>
</tr>
<tr>
<td>Cumulative dose of gonadotropins (IU)</td>
<td>1976 ± 730</td>
<td>2286 ± 845</td>
<td>3050 ± 2257</td>
<td>0.007</td>
<td>2156.4 ± 743.9</td>
</tr>
<tr>
<td>Mean duration of stimulation (days)</td>
<td>9.4 ± 2.4</td>
<td>10.6 ± 1.4</td>
<td>10.2 ± 2.4</td>
<td>0.20</td>
<td>9.3 ± 1.4</td>
</tr>
<tr>
<td>GnRH agonist trigger, n (%)</td>
<td>52 (93)</td>
<td>9 (90)</td>
<td>16 (89)</td>
<td>N/A</td>
<td>102 (90.3)</td>
</tr>
<tr>
<td>NCg trigger, n (%)</td>
<td>3 (5)</td>
<td>1 (10)</td>
<td>2 (11)</td>
<td>N/A</td>
<td>9 (8.0)</td>
</tr>
<tr>
<td>Dual trigger, n (%)</td>
<td>1 (2)</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>2 (1.7)</td>
</tr>
</tbody>
</table>

rFSH, recombinant FSH.

### Table IV
Number of metaphase II oocytes vitrified and number of livebirths (post-pubertal patients).

<table>
<thead>
<tr>
<th>FP procedure</th>
<th>n</th>
<th>Metaphase II oocytes (mean ± SD)</th>
<th>Number of livebirths in returning patients (n = 17) using cryopreserved material</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS</td>
<td>85</td>
<td>10.8 ± 7.1</td>
<td>4/9</td>
</tr>
<tr>
<td>Consecutive COS</td>
<td>11</td>
<td>18.2 ± 12.7</td>
<td>1/2</td>
</tr>
<tr>
<td>OPU-IVM</td>
<td>17</td>
<td>9.2 ± 10.1</td>
<td>0/1</td>
</tr>
<tr>
<td>OTC + OTO-IVM</td>
<td>25</td>
<td>4.1 ± 3.7</td>
<td>1/2</td>
</tr>
<tr>
<td>OPU-IVM + OTC + OTO-IVM</td>
<td>17</td>
<td>13.5 ± 9.7</td>
<td>1/2</td>
</tr>
<tr>
<td>OTC + OTO-IVM + COS</td>
<td>13</td>
<td>11.3 ± 6.6</td>
<td>1/1</td>
</tr>
<tr>
<td>Other combinations</td>
<td>26</td>
<td>9.1 ± 7.5</td>
<td>0/2</td>
</tr>
</tbody>
</table>

P-value (Kruskal–Wallis test)

MII, metaphase II.

### Table V
Number of metaphase II oocytes vitrified in patients with breast, haematological and gynaecological cancer.

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer (n = 56)</th>
<th>Haematological cancer (n = 10)</th>
<th>Gynaecological cancer (n = 18)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MII oocytes, mean ± SD (all procedures)</td>
<td>9.9 ± 7.9</td>
<td>8.7 ± 7.8</td>
<td>10.7 ± 10.1</td>
<td>0.57</td>
</tr>
<tr>
<td>MII oocytes, mean ± SD (COS only)</td>
<td>9.5 ± 6.2</td>
<td>14.5 ± 6.2</td>
<td>10.3 ± 7.0</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Comparison of the mean number of oocytes vitrified after fertility preservation. Results for the entire cohort and results for patients who had COS only are displayed separately.