Strategies to safely use cryopreserved ovarian tissue to restore fertility after cancer: a systematic review

BIOGRAPHY
Lotte Eijkenboom,(MSc graduated from the Faculty of Medicine at Radboud University in Nijmegen, the Netherlands, in 2017. She has since joined the Department of Reproductive Medicine at the Radboud University Medical Centre, Nijmegen. Her research focuses on enhancing the safety of autotransplantation of cryopreserved ovarian cortex for oncological patients.

Lotte Eijkenboom1,*, Emma Saedt1, Carlijn Zietse2, Didi Braat1, Catharina Beerendonk1, Ronald Peek1

KEY MESSAGE
Strategies to circumvent the reintroduction of cancer through ovarian tissue transplantation are being developed, but have not reached the stage of clinical trials. Further research is required to establish their potential risks and effectiveness. The key to improving these strategies is a deeper understanding of the process of oogenesis.

ABSTRACT
Ovarian tissue cryopreservation and subsequent autotransplantation is a successful technique for fertility preservation in oncological patients. However, there are concerns regarding safety, as the graft may contain malignant cells that could lead to the reintroduction of cancer. To circumvent this problem several experimental strategies are being pursued. This systematic review was conducted to provide an overview of the strategies aiming to safely use cryopreserved human ovarian tissue to restore fertility after cancer. Thirty-one studies were included, covering five different experimental strategies: (i) in-vitro maturation of oocytes, (ii) constructing an artificial ovary as a scaffold for reseeding pre-antral follicles, (iii) purging strategies aimed at the eradication of contaminating malignant cells, (iv) maturation of oocytes by xenotransplantation, and (v) stem cell-based oogenesis. These strategies to circumvent the reintroduction of cancer cells through ovarian tissue autotransplantation are being developed, but so far have not reached the stage of clinical trials. Further research is required to establish their risks and effectiveness while the ethical aspects associated with these strategies also need to be discussed. Despite the fact that these experimental procedures are still under development, they might provide safe fertility restoration options for oncological patients in the future.
INTRODUCTION

Ovarian tissue cryopreservation (OTC) is becoming an established fertility preservation technique that can be applied before the start of gonadotoxic therapy, especially for prepubertal girls and patients who require immediate treatment and do not have time for oocyte or embryo preservation (Donnez and Dolmans, 2017; Liebenthron et al., 2019). The technique has also proved to be more successful than many could have imagined. Worldwide, more than 200 births have been reported after the autotransplantation of frozen ovarian tissue (Dolmans et al., 2020). A well-documented series of ovarian tissue transplantations (OTT) from five European centres has shown that the chance of having one or more live births after a transplant is 26% (Dolmans et al., 2021). Another multicentre study even reported a 41.6% chance of at least one delivery after OTT (Shapiro et al., 2020).

With these developments, important professional organizations in the field of reproduction (American Society for Reproductive Medicine and European Society of Human Reproduction and Embryology) have stated that the technique has passed the experimental stage and should now be referred to as innovative and usual care. Nevertheless OTC still faces many challenges in clinical application, especially for cancer patients. The frozen ovarian tissue, which is generally stored before the start of cancer treatment or after the remission induction phase, carries the risk of harbouring metastasized malignant cells (Basting et al., 2013; Dolmans et al., 2013; Meiraw et al., 2008; Rosendahl et al., 2013). After thawing and subsequent transplantation of the ovarian tissue these micrometastases have the capacity to develop into tumours in a mouse model (Dolmans et al., 2010; Shaw et al., 1996), indicating that the autotransplantation of frozen-thawed ovarian tissue could lead to the reintroduction of the malignancy.

While OTT is possibly safe in most solid tumours because ovarian involvement is limited (Basting et al., 2013; Dolmans et al., 2016; Fabbri et al., 2012b), the ovarian tissue from patients with blood-borne malignancies, such as leukaemia, has up to a 50% chance of harbouring malignant cells (Dolmans et al., 2013, Shapiro et al., 2018b). The probability of reintroducing cancer cells is relatively small (Dolmans et al., 2021; Gellert et al., 2018) and the transplantation of a small number of cancer cells could be insufficient to lead to reintroduction of the malignancy (Soares et al., 2015b). However, even the slightest risk of reintroducing a malignant condition remains an alarming scenario. Transplantation of frozen-thawed ovarian tissue in women cured of malignancies with a high risk of ovarian involvement should thus only be performed in fertility clinics with extensive experience in OTT and after thorough consideration of the case by a multidisciplinary team. With the rising number of OTT, the urgency to develop strategies directed at the elimination of malignant cells or to offer alternatives using cryopreserved ovarian tissue to increase the safety of fertility restoration for oncological patients has become even more evident.

The possible contamination of ovarian cortex fragments with cancer cells should always be carefully evaluated before autotransplantation, and a range of different methods is being used to detect these metastasized cells. A patient tailor-made approach should be used whenever possible, including the (immuno)histochemistry of tumour-specific markers, molecular analysis of tumour-specific transcripts or xenotransplantation into immunodeficient mice to confirm the absence of metastatic cancer cells (Shapiro et al., 2018b; Shapiro et al., 2018b). However, all these tests are destructive to the ovarian tissue that is being analysed, and cannot be applied to the cortex fragments that will ultimately be transplanted (Diaz-Garcia et al., 2019; Hoekman et al., 2015; Soares et al., 2017). Even when the tested fragment is found to be devoid of malignant cells, the remaining fragments could still harbour micrometastases because there might be a sampling bias (Bockstaale et al., 2012; Diaz-Garcia et al., 2019; Rosendahl et al., 2010). Strategies to safely use cryopreserved ovarian tissue for fertility restoration after cancer are therefore needed, especially for women considered to have a high risk of ovarian metastases.

This has spurred the development of innovative techniques to prevent the reintroduction of malignant cells during autotransplantation and will ultimately lead to a safer and more widely applicable range of fertility preservation strategies. The authors have identified five different strategies that are directed at preventing the reintroduction of malignant cells after OTC: (i) in-vitro maturation (IVM) of oocytes from the ovarian cortex to perform IVF, (ii) the development of an artificial ovary, (iii) purging ovarian tissue of contaminating malignant cells, (iv) maturation of oocytes by xenotransplantation of ovarian cortex tissue, and (v) stem cell-based oogenesis. These strategies are still in the experimental stage and have therefore not yet been introduced into daily practice. This systematic review provides a comprehensible overview of these strategies and discusses the stage of development, the advantages and disadvantages of the various strategies, their possible applicability in clinical practice and recommendations for future research.

MATERIALS AND METHODS

Protocol and registration

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al., 2021) and the protocol was registered on the International Prospective Register for Systematic Reviews (PROSPERO, Registration ID CRD42020197284).

Search strategy and selection criteria

MEDLINE (using the PubMed database), EMBASE and the Cochrane Library were searched for eligible studies on 8 July 2021. The search strategy (Table 1) was formulated after consultation with an information specialist from Radboud University Nijmegen Library, and combinations of Medical Subject Headings (MeSH) or Emtree terms and free text words were used. Articles were selected for inclusion in the systematic review if they fulfilled the following criteria: original studies aiming for the safe restoration of fertility in oncological patients after OTC, publications that pertained to experiments with human ovarian tissue, and studies published in English and from 1 January 2000 and onwards. A cut-off date of 2000 was applied as the first human autotransplantation of frozen-thawed ovarian tissue was reported that year (Oktay and Kartikaya, 2000). The search was thus restricted to articles
Type III studies: purging of ovarian tissue

Type IV studies: other techniques

<table>
<thead>
<tr>
<th>Type I studies: in-vitro maturation of oocytes</th>
<th>Type II studies: artificial ovary</th>
<th>Type III studies: purging of ovarian tissue</th>
<th>Type IV studies: other techniques</th>
</tr>
</thead>
</table>

Data extraction

The following data were extracted from all the included studies: the author of references and reviews (FIGURE 1). After the removal of duplicates 8914 records were screened, based on the title and abstract that matched the study’s criteria. Of those, 166 full-text articles were considered potentially eligible, of which 31 were included in the qualitative synthesis. The included studies dated from 2004 to 2021. The studies were grouped according to the strategy used to safely restore fertility after cancer using cryopreserved ovarian tissue. Five different experimental strategies to prevent the reintroduction of malignancy were identified: (i) IVM of oocytes isolated from the ovarian cortex to perform IVF; (ii) construction of an artificial ovary to reseed pre-antral follicles, (iii) purging strategies aimed at the elimination of contaminating malignant cells from the ovarian cortex tissue, (iv) maturation of oocytes by xenotransplantation of ovarian cortex, and (v) stem cell-based oogenesis (FIGURE 2).

IVM of oocytes

In-vitro-matured oocytes could potentially be fertilized through IVF. The resulting embryos could then be transferred to the patient without the risk of reseeding malignant cells as there is no need for autotransplantation of the intact ovarian cortex tissue. IVM is performed on oocytes collected by three different procedures: (i) IVM of small follicles isolated from ovarian tissue fragments, (ii) IVM of cumulus-oocyte complexes from small antral follicles or spent media collected during the preparation of ovarian tissue fragments for cryopreservation (Fasano et al., 2017; Kedem et al., 2018), and (iii) IVM of immature oocytes harvested from small antral follicles by transvaginal ovum retrieval (Son et al., 2019). This systematic review will focus only on the first-mentioned technique based on oocytes from ovarian tissue obtained by ovariectomy because this is the technique that can be used to perform IVM of oocytes from already stored cryopreserved ovarian tissue. Several culture systems have been developed for the IVM of oocytes from isolated follicles or intact ovarian tissue fragments, occasionally followed by follicle isolation and in-vitro growth for further oocyte maturation (TABLE 2).

As different oocyte developmental stages require different maturation techniques, various culture steps have been developed and combined to obtain mature oocytes. Telfer and colleagues reported a two-step culture system showing that human unilateral follicles were activated in fragmented fresh cortical tissue (Telfer et al., 2008). During the second step of this culture system the secondary follicles were isolated from the tissue. Additional culture in the presence of activin A led to a further increase in size of these secondary follicles.
follicles. The other recent successful IVM of oocytes from unilaminar follicles was achieved by combining even more culture steps (McLaughlin et al., 2018). In this multistep culture system fresh ovarian cortex tissue was fragmented and the primordial and primary follicles were allowed to grow to secondary follicles while still embedded in the ovarian cortex tissue. The secondary follicles were manually dissected from the tissue and these individual follicles were subsequently cultured in the presence of activin A and FSH. This last culture step eventually resulted in cumulus–oocyte complexes containing metaphase II (MII) oocytes. However, these oocytes produced atypical large polar bodies with a ratio of 4:1 to 3:1 of oocyte size to polar body size, and the degree of cumulus expansion was less when compared with in-vivo matured oocytes.

An alternative approach to IVM is the culture of isolated pre-antral follicles in three-dimensional alginate matrices. A matrix provides pre-antral follicles with

FIGURE 1 PRISMA flow chart of the systematic review process.

FIGURE 2 Overview of fertility restoration techniques to improve safety after autotransplantation of cryopreserved ovarian tissue in oncological patients.
### TABLE 2 DATA FROM TYPE I STUDIES: IN-VITRO MATURATION OF SMALL FOLLICLES

<table>
<thead>
<tr>
<th>Authors (year)</th>
<th>Tissue source</th>
<th>Starting follicle stage</th>
<th>Study design a</th>
<th>Outcomes: final achieved maturation stage, hormone production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tellier et al. (2008)</td>
<td>Fresh ovarian cortex tissue from women undergoing Caesar-ean section</td>
<td>Primordial, transitional and primary follicles</td>
<td>1. Two-step culture system with ovarian fragment cultured in McCoy's 5a culture medium followed by isolated follicle culture with activin A; 2. 5 days' culture of the fragment; 3. isolated follicles; 4. Follicle culture without activin; 5. 6 women; 96 tissue fragments</td>
<td>Pre-antral follicles increased more in size (143 ± 174 µm) compared with the control group (P &lt; 0.005); 90% of the pre-antral follicles cultured with activin A increased in size during the first 2 days versus 36% in the control group (P &lt; 0.005)</td>
</tr>
<tr>
<td>Amanim et al. (2009)</td>
<td>Cryopreserved ovarian cortex tissue, not further specified</td>
<td>Pre-antral stage</td>
<td>1. Isolated follicles cultured in calcium alginate (1%) matrix; 2. 7 days; 3. 4 control groups: after biopsy, freezing, follicle isolation and alginate embedding; 4. 4 women; 159 follicles</td>
<td>All follicles increased in size (final size 56.73 ± 13.10 µm); survival rate 90%, follicles remained in the pre-antral stage</td>
</tr>
<tr>
<td>Xu et al. (2009)</td>
<td>Fresh ovarian cortex tissue from women with cancer</td>
<td>Pre-antral to early antral stage</td>
<td>1. Isolated follicles cultured in 0.5% alginate beads diluted Matrigel matrix; 2. 30 days; 3. –; 4. 14 women; Matrigel n = 18 follicles; alginate n = 22 follicles</td>
<td>Oocyte reached antral stage; growth increased from 75 to 110 µm; follicles produced increasing levels of 17β-oestradiol and progesterone on culture days 5 to 30</td>
</tr>
<tr>
<td>Fabbri et al. (2012)</td>
<td>Cryopreserved ovarian cortex tissue from a girl with Wilms tumour</td>
<td>Not specified</td>
<td>1. Ovarian tissue fragments cultured in α-MEM culture medium with FSH and LH; FSH and LH concentrations were tripled once a month; 2. 8, 24 and 32 weeks; 3. α-MEM without FSH and LH; 4. Tissue from 1 patient, number of fragments not specified</td>
<td>A cluster of growing pre-antral follicles with 2 prematurally oocytes in the group with gonadotropins versus 1 degenerating pre-antral follicle in the control group</td>
</tr>
<tr>
<td>Khosravi et al. (2013)</td>
<td>Cryopreserved ovarian tissue from women with benign gynaecological conditions</td>
<td>Primordial, transitional, primary and secondary follicles</td>
<td>1. Ovarian tissue fragments cultured in McCoy's 5a culture medium; 2. 7 days; 3. Fresh ovarian tissue strips; 4. 6 women; 12 vitrified strips; 12 control strips</td>
<td>Percentage of secondary/pre-antral follicles after culture increased from 20.93% to 38.64% (P &lt; 0.05) in the vitrified group versus an increase from 25% to 56% in the fresh group (P &lt; 0.05)</td>
</tr>
<tr>
<td>Xia et al. (2015)</td>
<td>Cryopreserved ovarian tissue from women with benign and malignant gynaecological conditions</td>
<td>Primary and secondary follicles</td>
<td>1. Isolated follicles cultured in 3-dimensional alginate beads (1% alginate) with human MSC; 2. 8 days; 3. Alginate beads without MSC; 4. 7 women; 91 follicles</td>
<td>Growth rates after culture with MSC were 58–82.5% versus 27% in the control group (P &lt; 0.05), follicles remained in the pre-antral stage; follicle oestradiol production was increased compared with the controls (P &lt; 0.05)</td>
</tr>
<tr>
<td>Xiao et al. (2015)</td>
<td>Ovarian cortex tissue from women with cancer (cryopreserved/fresh not specified)</td>
<td>Multilayer second- ary follicles</td>
<td>1. Isolated follicles cultured in alginate–hydrogel encapsulation (0.5% alginate) followed by basic medium culture; 2. 30–40 days; 3. Culture only in alginate; 4. 44 women; 65 follicles</td>
<td>Meiotically competent MII oocytes were obtained (20% of the follicles); oocytes in the control group remained in germinal vesicle stage or degenerated; 17β-oestradiol, progesterone and AMH increased during follicle maturation</td>
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<tr>
<td>Younis et al. (2017)</td>
<td>Cryopreserved ovarian cortex tissue from girls/women with benign and malignant conditions</td>
<td>Primordial follicles</td>
<td>1. Ovarian tissue fragments cultured in 4 different matrices (hrVit, SIS, alginate and CollPlant) with LIF; 2. 6 days; 3. Matrices without LIF; 4. 20 women; 35 samples</td>
<td>Primordial follicle growth was not enhanced by LIF, there was no significant difference in follicle growth between the different matrices; higher concentrations of 17β-oestradiol in medium after culture on CollPlant and alginate; higher concentrations of AMH in medium after culture on hrVit and SIS</td>
</tr>
<tr>
<td>McLoughlin et al. (2018)</td>
<td>Fresh ovarian cortex tissue from healthy women</td>
<td>Primordial, transitional and primary follicles</td>
<td>1. Multistep culture system: culture of ovarian strips, isolation of secondary follicles with activin A, retrieval of COC and cultured with activin A and FSH; &gt;100 µm oocytes cultured in SAGE medium; 2. 21 days; 3. –; 4. 10 women; 160 tissue fragments; 87 follicles</td>
<td>54/87 secondary follicles reached the antral stage; 32/48 COC contained oocytes &gt;100 µm; 9/32 secondary follicles reached the MII stage</td>
</tr>
<tr>
<td>Xu et al. (2021)</td>
<td>Fresh ovarian cortex tissue from women with benign gynaecological conditions</td>
<td>Primordial follicles</td>
<td>1. Culture of ovarian tissue fragments in culture medium followed by isolation of secondary follicles with or without AMH modulation; 2. 3 weeks followed by 6 weeks isolated follicle culture with or without AMH; 3. –; 4. 13 women; 40 tissue fragments</td>
<td>Increase in AMH concentrations (P &lt; 0.05) in culture media; growth to the antral stage during 6 weeks of individual culture; increased media concentrations of 17β-oestradiol, activin A, IGF2 and VEGF in the group with AMH modulation; progression to MII oocytes in 21.4% of secondary follicles</td>
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</tbody>
</table>

a Study design: 1, culture system; 2, culture period; 3, control group; 4, sample size. AMH, anti-Mullerian hormone; COC, cumulus–oocyte complex; CollPlant, human recombinant virgin collagen bioengineered in tobacco plant lines; hrVit, human recombinant vitronectin; IGF2, insulin-like growth factor 2; LIF, leukaemia inhibitory factor; MII, metaphase II; MSC, Mesenchymal stem cells; SIS, small intestine submucosa.
the physical support to maintain their structure, resulting in follicle growth (Amorim et al., 2009; Xia et al., 2015; Xu et al., 2009) and oocyte maturation to the MII stage (Xiao et al., 2015). Follicular growth and maturation of the oocytes in these studies also resulted in increased 17β-estradiol, progesterone and anti-Müllerian hormone (AMH) concentrations in the spent medium (Xia et al., 2015; Xiao et al., 2015; Xu et al., 2009; Xu et al., 2021). Not only did hormone production resume in the follicles after the IVM experiment, but the addition of AMH to the culture medium of isolated secondary follicles followed by AMH depletion increased the production of steroid hormones and paracrine factors, and this led to the formation of MII oocytes (Xu et al., 2021).

However, primordial follicle activation could be observed even without a multistep culture system, matrix or hormonal stimulation. The spontaneous follicle activation was most probably due to the disruption of the Hippo signalling pathway during tissue preparation and subsequent culturing (Febbri et al., 2012a; Khasrov et al., 2013; Younis et al., 2017). IVM of oocytes from human ovarian tissue through spontaneous follicle activation, multistep culture systems, matrices or hormonal stimulation has not yet led to a complete meiotically competent oocyte that can be used for IVF.

**The artificial ovary**

Developing an artificial ovary could serve several purposes including the construction of a support system for isolated ovarian follicles devoid of malignant cell contamination. The artificial ovary may then be transplanted to the remaining ovary or pelvic region in the former cancer patient or could be cultured ex vivo to obtain mature oocytes for IVF (Figure 2). This strategy relies on isolated follicles and might be safe because ovarian follicles are encapsulated by a basal membrane that shields them from blood capillaries, stromal cells and possibly also contaminating malignant cells. The artificial ovary itself needs to mimic the ovarian cortex microenvironment with enough support and rigidity to promote the growth of the encapsulated follicles.

Several methods using synthetic biomaterials to support the maturation of oocytes have been investigated, including matrices composed of human plasma clots (Dolmans et al., 2007; Dolmans et al., 2008), agarose (Krotz et al., 2010), Matrigel (Laronda et al., 2014) or fibrinogen/thrombin combinations (Chiti et al., 2018; Luycx et al., 2013; Paulini et al., 2016), encapsulation in alginate beads (Laronda et al., 2014; Yin et al., 2016) and even decellularized human ovarian tissue (Pors et al., 2019) to sustain the follicular structure throughout the oocyte maturation process (Table 3).

Dolmans and colleagues opted for embedding purified isolated follicles in human plasma clots followed by xenotransplantation to mice. In this artificial matrix, the small follicles were able to develop to secondary and antral follicles (Dolmans et al., 2007; Dolmans et al., 2008). In the study by Krotz co-workers theca and granulosa cells from antral follicles were seeded in micro-moulded agarose gels and formed a 3D microtissue (Krotz et al., 2010). The cumulus–granulosa–oocyte complexes embedded in the microtissue formed large spheroids and showed polar body extrusion. However, since the study by Krotz and co-workers used oocytes from antral follicles for their 3D microtissue, the outcome of the maturation stage cannot be compared with other studies using oocytes from pre-antral follicles (Table 3).

In contrast to 3D microtissue with agarose gels, artificial ovaries composed of alginate beads showed no survival of primordial follicles but supported only the growth of secondary follicles (Laronda et al., 2014; Yin et al., 2016).

Over the years, ongoing research on matrices composed of different combinations of fibrinogen and thrombin led to an improved follicle recovery rate of up to 47% (Chiti et al., 2018; Luycx et al., 2013; Paulini et al., 2016). Optimal concentrations and ratios of fibrinogen/thrombin differed considerably between studies. Paulini and collaborators demonstrated a viability rate of 100% after a 7-day culture in 50 mg/ml fibrinogen and 10 IU/ml thrombin (F50/T10) (Paulini et al., 2015a). Luycx and colleagues, however, recommended F12.5/T1 and F25/T4 because of the reproducible degradation of the fibrin network, the increased survival and proliferation of oocytes, the support for stromal cells and the increase of stromal cell density (Luycx et al., 2013). More recently, Chiti and co-workers reported optimal fibrinogen/thrombin combinations to be F50/T50, because the ultrastructure and rigidity of this matrix most closely resembles that of human ovarian cortex (Chiti et al., 2018).

In addition to these completely artificial matrices the possibility of using decellularized human ovarian cortex tissue for the construction of an artificial ovary was also explored (Pors et al., 2019). After careful elimination of all cellular components, including possible contaminating malignant cells, this matrix may provide a more natural scaffold for isolated follicles compared with artificially constructed networks. Follicles reseeded with Matrigel were shown to remain viable in the decellularized tissue for up to 3 weeks after xenografting (Pors et al., 2019). Although there are promising results regarding the use of an artificial ovary to overcome the problem of cancer cell reintroduction, a more profound understanding of the ovarian cortex tissue structure will help guide the design and development of an artificial ovary able to support the complex processes involved in folliculogenesis.

**Purging ovarian cortex**

Ex-vivo treatment of ovarian cortex fragments or cell suspensions to eradicate contaminating malignant cells prior to autotransplantation has also been explored as an option to prevent the reintroduction of cancer. This strategy, also known as purging, relies on the use of specific inhibitors or pharmacological steps that eliminate the contaminating cancer cells but do not impair the functional integrity of the ovarian follicles or stromal cells. Several research groups have been focusing on purging as a method to eliminate malignant cells from ovarian tissue using various protocols (Table 4).

One approach to purging is dissociation of the ovarian cortex tissue to eliminate tumour cells from the cell suspension (Schröder et al., 2004; Soares et al., 2015a; Soares et al., 2017). Breast cancer tumour cells added to ovarian tissue in suspension were largely, but not completely, eliminated after purging with activated human lymphocytes and the bispecific antibody BiS-1, while the follicles remained morphologically intact (Schröder et al., 2004). Soares and collaborators established efficient purging by conducting several consecutive washes of follicles from ovarian tissue suspensions from healthy patients that were artificially contaminated with leukaemia cells (Soares et al., 2015a). To
<table>
<thead>
<tr>
<th>Authors (year)</th>
<th>Tissue source</th>
<th>Matrix type</th>
<th>Study design*</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolmans et al. (2007)</td>
<td>Fresh ovarian cortex tissue and isolated follicles from women with benign gynaecological conditions</td>
<td>Human plasma clots</td>
<td>1. Isolated follicles in plasma clot to the ovarian bursa of nude mice 2. Xenotransplantation to the ovarian bursa of nude mice for 7 days 3. Not grafted ovarian tissue and grafted ovarian tissue fragments 4. 9 patients</td>
<td>20.3% of isolated follicles were recovered; 0.3 secondary follicles in grafted clots; stroma-like tissue formed around the isolated follicles</td>
</tr>
<tr>
<td>Dolmans et al. (2008)</td>
<td>Isolated follicles from fresh ovarian cortex of women with benign gynaecological conditions</td>
<td>Human plasma clots</td>
<td>1. Isolated pre-antral follicles in plasma clots 2. Xenotransplantation to ovarian bursa of SCID mice for 24 weeks 3. – 4. 3 women, 487 follicles</td>
<td>29% of the follicles were recovered; 36% of the follicles recovered from the graft had reached the secondary stage and 4.8% the antral stage</td>
</tr>
<tr>
<td>Krotz et al. (2010)</td>
<td>Fresh ovarian cortex tissue from women with benign gynaecological conditions</td>
<td>Micro-moulded agarose gels</td>
<td>1. Isolated ovarian theca and granulosa cells and COC from antral follicles 2. 72 h co-culture of theca cells and COC 3. – 4. Not described</td>
<td>1/3 of the oocytes had polar body extrusion after 48 h of culture. After 72 h large spheroids were formed</td>
</tr>
<tr>
<td>Luiczk et al. (2013)</td>
<td>Fresh ovarian cortex tissue from women with benign gynaecological conditions</td>
<td>Fibrinogen/thrombin matrix; 9 combinations tested (F1/T4, F12.5/T1, F12.5/T20, F25/T1, F25/T20, F50/T1, F50/T20 and F100/T)</td>
<td>1. Isolated ovarian stromal cells 2. Isolated ovarian cells 22 days in culture medium, 1 week in matrix 3. Uncultured fresh ovarian cortex 4. 4 women; two samples from each woman per F/T combination</td>
<td>F12.5/T1 and F25/T4 were the optimal combinations based on reproducible degradation of the fibrin network, survival and proliferation analyses, good support for stromal cells and increase of stromal cell density</td>
</tr>
<tr>
<td>Laronda et al. (2014)</td>
<td>Fresh ovarian cortex and medulla from women with malignant diseases</td>
<td>0.5% or 2% alginate beads for primordial follicles; Matrigel matrix for secondary follicle isolation; 0.25%, 0.5%, 1% or 2% alginate for cortical tissue pieces</td>
<td>1. Isolated primordial and secondary follicles or cortical tissue pieces 2. 3 days for primordial follicles, 21 days for secondary follicles or 1–6 weeks for cortical tissue pieces 3. Uncultured fresh ovarian cortex 4. 32 women; two samples from each woman per F/T combination</td>
<td>No survival of isolated primordial follicles. Growth of isolated secondary follicles. No difference in survival of cortical tissue pieces with different alginate concentrations; survival was 100%, 85.4%, 74.1%, 52.8% and 46.67% following 1, 2, 3, 4 and 6 weeks of culture. A higher percentage of growing follicles after more than 2 weeks of culture</td>
</tr>
<tr>
<td>Paulini et al. (2016)</td>
<td>Frozen-thawed ovarian cortex tissue from women with benign gynaecological conditions</td>
<td>Fibrinogen/thrombin matrix clots; F50/T10; with or without 3% hyaluronic acid</td>
<td>1. Isolated pre-antral follicles and ovarian stromal cells 2. 7 days of xenotransplantation in mice 3. – 4. 10 women; 8 clots, 30–50 follicles and 50,000 stromal cells per clot</td>
<td>100% of follicles in both groups were viable after day 7; follicle recovery rate in clots with hyaluronic acid was 20.5% versus 23.4% without hyaluronic acid (statistically not significant). Higher proportion of secondary follicles without hyaluronic acid versus with (P &lt; 0.05)</td>
</tr>
<tr>
<td>Yin et al. (2016)</td>
<td>Fresh ovarian cortex tissue and medulla from women with malignant diseases</td>
<td>0.3% or 0.5% alginate beads encapsulated with or without ovarian interstitial tissue fragments</td>
<td>1. Viable isolated follicles and ovarian interstitial tissue fragments 2. &gt;30 days in matrix 3. – 4. 16 women; 395 follicles</td>
<td>15% survival for primary follicles and 60% for secondary follicles. Final average diameter of secondary follicles was 377 μm. No difference with or without interstitial tissue fragments</td>
</tr>
<tr>
<td>Chi et al. (2018)</td>
<td>Fresh and frozen-thawed ovarian cortex tissue from women with benign gynaecological conditions</td>
<td>Fibrinogen/thrombin matrix; 4 combinations tested (F12.5/T1, F30/T50, F50/T50, F75/T75)</td>
<td>1. Isolated pre-antral follicles 2. 24 h 3. – 4. 22 women; 696 follicles in total</td>
<td>Follicle recovery and loss rates ranged 44–47% and 29–39%, respectively. No statistical difference was noted, F50/T50 appears to be the optimal combination and most closely resembles human ovarian cortex</td>
</tr>
<tr>
<td>Soares et al. (2017)</td>
<td>Frozen-thawed ovarian cortex and medulla from women with malignant diseases</td>
<td>Decellularized human ovarian tissue</td>
<td>1. Isolated pre-antral follicles with Matrigel and ovarian stromal cells 2. First 4 weeks of culture in vitro, then 2–3 weeks of xenotransplantation in mice 3. DCT without follicles 4. 21 women; 20 cultured and xenotransplanted follicles</td>
<td>Ovarian stroma cells were able to recellularize the DCT, 12/20 pre-antral follicles remained viable after 4 weeks culture; follicle recovery rate of 25% after xenotransplantation with Matrigel</td>
</tr>
</tbody>
</table>

* Study design: 1, cells used for culture; 2, culture period; 3, control group; 4, sample size.
COC, cumulus-oocyte complex; DCT, xxxx; F/T, fibrinogen (mg/ml)/thrombin (IU/ml); DCT, decellularized human ovarian tissue; SCID, severe combined immunodeficient.

Confirm complete purging the experiment was repeated with ovarian tissue from leukaemia patients (Soares et al., 2017). While only a single leukaemia cell was detected after the follicle washes in the experiment with artificially induced leukaemia cells, there were none in the ovarian tissue suspension of leukaemia patients. These approaches to purging have the potential to provide isolated follicles free of malignant cells that could be encapsulated in an transplantable artificial ovary.

In addition to purging of ovarian cortex cell suspensions, intact ovarian cortex fragments can be subjected to purging to eradicate contaminating cancer cells (Soares et al., 2019; Eijkenboom et al., 2021; Mulder et al., 2019). In contrast to cell suspensions, a purged intact ovarian cortex fragment can be directly used for autotransplantation. Ovarian
No leukaemia cells could be detected in follicle suspension after three washes versus 23% (SD 15%; P < 0.001) in the control group. For frozen-thawed human ovarian tissue xenotransplantation, a low yield of MII oocytes was reached in mice followed by IVM of aspirated follicles after three washes versus 95.6% viable follicles in the control group. No statistically significant difference (P = 0.9)

<table>
<thead>
<tr>
<th>Authors (year)</th>
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<th>Study designa</th>
<th>Detection method for residual disease</th>
<th>Outcomes: purging efficiency</th>
<th>Viability tests after purging</th>
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<tr>
<td>Schröder et al. (2004)</td>
<td>Frozen-thawed ovarian tissue in suspension from two patients with malignancy and one with a BRCA1 mutation</td>
<td>1. BIS-1 and activated lymphocytes 2. MCF-7 breast cancer cells 3. Ovarian tissue suspension with T-cells without BIS-1 4. 3 patients</td>
<td>Fluorescent microscopy and MTT assay</td>
<td>A maximum depletion of growing MCF-7 cells of 89% (SD 11%) after BIS-1 treatment versus 23% (SD 15%; P &lt; 0.001) in the control group</td>
<td>Morphology: follicles remained intact</td>
</tr>
<tr>
<td>Soares et al. (2015a)</td>
<td>Fresh ovarian cortex tissue in suspension from women with benign gynaecological conditions</td>
<td>1. Three washes in droplets of PBS + 10% FBS with follicle pick-up 2. BV-173 leukaemia cells added to ovarian cell suspension 3. Follicle retrieval technique without washes 4. 10 patients, 772 follicles</td>
<td>Fluorescent microscopy and PCR analysis (BCR-ABL)</td>
<td>1 leukaemia cell among 772 follicles after purging versus 194 leukaemia cells among 499 retrieved follicles in controls; BCR-ABL was not present in purged follicle suspension but was present in 4/7 control samples</td>
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<td>PCR analysis</td>
<td>No leukaemia cells could be detected in follicle suspension after three washes</td>
<td>Calcein AM/ethidium homodimer viability assay: total good-quality viable follicle rate was 92.06%</td>
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<tr>
<td>Mulder et al. (2019)</td>
<td>Frozen-thawed ovarian cortex tissue from transgenders</td>
<td>1. Verteporfin with or without imatinib 2. Artificially induced rhabdomyosarcoma, Ewing sarcoma, breast cancer or CML 3. Equivalent solvent concentration 4. 8 patients</td>
<td>Histology and PCR analysis</td>
<td>RD, Rh36 (rhabdomyosarcoma) and K562 (CML) cells were completely purged; markers of MCF-7 (breast cancer) and ES-2 (Ewing sarcoma) cells were still detectable</td>
<td>Morphology, glucose-uptake, neutral red uptake and follicular apoptosis showed no statistically significant difference (P &gt; 0.05) between the verteporfin-treated and control groups</td>
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<td>Diaz-Garcia et al. (2019)</td>
<td>Frozen-thawed ovarian cortex tissue from leukaemia patients</td>
<td>1. DXM 2. Acute lymphoblastic leukaemia, naturally metastasized 3. Basal media 4. 6 patients, 11 ovarian fragments in DXM and control groups</td>
<td>Xenografting into mice for 6 months followed by histology and PCR analysis</td>
<td>Leukaemia cells were detected in 5/11 DXM-treated fragments and in 1/11 control fragments by PCR</td>
<td>Histology: healthy primordial, primary and secondary follicles regardless of treatment</td>
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<td>Eijkenboom et al. (2021)</td>
<td>Frozen-thawed ovarian cortex tissue from transgenders</td>
<td>1. GSK1090716 2. Artificially induced CML or AML 3. Equivalent solvent concentration 4. 8 patients</td>
<td>Histology</td>
<td>Formation of syncyta, no normal AML cells and CML cells (Jurkat-K562) detected</td>
<td>Morphology, glucose uptake, neutral red uptake, follicular apoptosis and in-vitro growth assay showed no difference between the GSK1090716 and control groups</td>
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*a Study design: 1, purging substance; 2, target cells; 3, control group; 4, sample size.

AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; DXM, dexamethasone; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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Cortex containing rhabdomyosarcoma or chronic myeloid leukaemia cells were effectively purged after a 24 h incubation with verteporfin. However, Ewing sarcoma and breast cancer cells persisted in the tissue after this treatment, indicating that different malignancies require different purging regimens (Mulder et al., 2019). Purging ovarian cortex tissue fragments with the Aurora kinases inhibitor GSK1090716 was shown to have a detrimental effect on contaminating chronic and acute myeloid leukemia cells by inducing large-scale apoptosis and formation of multinuclear syncyia (Eijkenboom et al., 2021). These pharmacologically based purging techniques were shown to have no effect on ovarian tissue metabolism, follicular morphology, follicular viability (Mulder et al., 2019) or in-vitro growth of small follicles to secondary follicles (Eijkenboom et al., 2021). In contrast, dexamethasone was found to be unable to effectively remove contaminating acute lymphocytic leukemia cells from ovarian cortex, and leukaemia cells could still be detected after xenotransplantation of the purged tissue fragments to mice (Diaz-Garcia et al., 2019). Although the pharmacological purging of contaminated ovarian cortex fragments shows promising results, long-term xenografting experiments are needed to confirm their efficacy and safety. Oocyte maturation through xenotransplantation Xenotransplanted frozen-thawed ovarian cortex tissue could be used as a source of purified mature oocytes suitable for IVF, thereby circumventing the problem of cancer cell transmission via the graft (TABLE 5). Multiple studies have shown that xenotransplantation of ovarian tissue from different animal species results in live offspring (Dittrich et al., 2015). For frozen-thawed human ovarian tissue the development of MII oocytes was reported after xenotransplantation and gonadotrophin stimulation of grafted mice followed by IVM of aspirated metaphase I oocytes, although only a low yield of MII oocytes was reached in these studies (TABLE 5) (Kim et al., 2005; Lotz et al., 2014b; Solemani et al., 2010). Even without external gonadotrophin stimulation ovarian grafts were able to form secondary follicles in mice, although
the yield of MII oocytes without hormone stimulation of the grafted mice again was relatively low (Lotz et al., 2014a).

In addition to the effect of hormonal stimulation of follicles after xenotransplantation, the effects of using different grafting sites in mice was also studied. From these experiments it was concluded that transplants into the back muscle supported considerably more MII oocyte formation compared with tissue grafted under the kidney capsule (17 versus 7 MII oocytes) (Soleimani et al., 2010).

Kim and colleagues studied the structure by immunocytochemical assessment of microtubules and DNA of MII oocytes after xenotransplantation of ovarian tissue and showed that some oocytes displayed abnormal nuclear and cytoplasmic maturation. Whether these abnormal MII oocytes were the result of damage sustained during the freeze-thaw process or by the gonadotrophin stimulation and xenotransplantation of the tissue remains unclear.

All of the included studies (TABLE 5) indicate that the xenotransplantation of human ovarian tissue with or without gonadotrophin stimulation may provide antral or MII oocytes, albeit with low efficacy.

### Stem cell-based oogenesis

Stem cells are promising instruments to improve fertility treatments in women after gonadotoxic therapy because of their intrinsic regenerative ability. The ultimate goal of stem cell-based oogenesis described in the included studies is to obtain fertilizable oocytes in vitro directly from stem cells harvested from ovarian tissue (TABLE 4). Parte and colleagues identified some of the cells obtained from the ovarian surface epithelium as very small embryonic-like stem cells (VSEL). These VSEL were capable of spontaneous differentiation in culture and formed oocyte-like cells of 130 µm in diameter with a distinct zona pellucida and extrusion of a polar body (Porte et al., 2011). Upon stimulation of ovarian tissue with FSH or basic fibroblast growth factor, ovarian stem cells also spontaneously differentiated to oocyte-like cells with an increased expression of gene transcripts specific for VSEL, ovarian germ stem cells (OGSC) and early germ cells (Porte et al., 2013). The increased expression of markers for oocyte and granulosa cell transition (GDF-9, LH-B and AMH) is suggestive of primordial follicle transition into primary follicles.

These studies indicate that the ovarian surface epithelium harbours VSEL that may provide an adequate source for further experiments on stem cell-based oogenesis, potentially offering a new strategy for treatment of female infertility.

### DISCUSSION

Even before the first transplantation of cryopreserved human ovarian tissue in 1999 (Oktay and Karlikaya, 2000) multiple strategies were pursued to provide safe autotransplantation of cryopreserved ovarian tissue without the risk of reintroducing malignant cells. Ovarian fragments were not only extensively screened to confirm the absence of malignant cells, but also xenografted to examine the risk of different malignancies (Bastings et al., 2013). Currently, ovarian cortex fragments are carefully evaluated prior to autotransplantation with an approach that is tailor-made for the patient whenever possible to decrease the risk of tumour cell transmission via the graft.
The risk of reintroducing malignancy in former cancer patients should be evaluated by a multidisciplinary team and performed in a fertility clinic with experience in OTT. So far, this approach has proved to be successful in patients with various malignancies (Dolmans et al., 2021; Shapiro et al., 2020). Furthermore, new experimental strategies are still being developed to enhance the safety of OTT, for example purging of ovarian tissue from malignant cells, the development of a transplantable artificial ovary, and restoration of fertility without autotransplantation such as IVM of oocytes from primordial follicles, an artificial ovary to enhance in-vitro growth and IVM, xenotransplantation to harvest mature oocytes and stem cell-based oogenesis. The characteristics of all these strategies directed at increasing the safety of autotransplantation of ovarian cortex tissue are summarized in Table 7 and discussed in more detail below.

Several culture systems have been developed to support the in-vitro growth of human follicles and maturation of oocytes. Although some research groups have been more successful than others, to date only technically demanding multistep culture systems have resulted in the growth of primordial follicles and the maturation of oocytes to the MII stage (Table 7) (McLaughlin et al., 2018; Xu et al., 2021). The challenges of IVM mainly lie in the complexity of the folliculogenesis process. Follicular growth and oocyte maturation are dependent on multiple facets including changing paracrine and autocrine factors, oocyte–somatic cell interactions, cytoskeleton stability and biomechanical forces (Lonergan and Fair, 2016; Telfer and Andersen, 2021). Consequently, the ex-vivo maturation of oocytes requires different culture conditions to support the various stages of follicle growth and oocyte maturation. For example, IVM requires a viable ongoing culture of ovarian tissue or isolated follicles, but also growth activation of the primordial follicles, manual dissection of the tissue to obtain secondary follicles for further development and finally maturation of oocyte–cumulus complexes (Telfer and Andersen 2021). Meeting all of these different conditions during the IVM process has proven to be difficult as well as technically demanding (McLaughlin et al., 2018). The changes in culture conditions during multistep IVM require specialized equipment and technical

<table>
<thead>
<tr>
<th>Parameter</th>
<th>In-vitro maturation</th>
<th>Artificial ovary</th>
<th>Purging</th>
<th>Xenotransplantation</th>
<th>Stem cell-based oogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reached maturation stage</td>
<td>Metaphase II oocytes</td>
<td>Metaphase II oocytes</td>
<td>Secondary follicles</td>
<td>Metaphase II oocytes</td>
<td>Oocyte-like cells expressing oocyte markers</td>
</tr>
<tr>
<td>Yield of oocytes</td>
<td>10.3–21.4% of the secondary isolated follicles formed oocytes with a polar body</td>
<td>3.6–60.0% of the follicles developed to the secondary or antral stage</td>
<td>79.0–96.4% of the follicles were viable after purging but no reports of yield of oocytes per maturation stage</td>
<td>0.1–0.46 antral follicles per graft</td>
<td>Not reported</td>
</tr>
<tr>
<td>Oocyte morphology</td>
<td>Abnormal large polar bodies</td>
<td>No abnormalities reported</td>
<td>No abnormalities reported</td>
<td>Slightly clumpy meiotic spindle and not all of the chromatin affixed to the metaphase plate</td>
<td>Cells of 130 μm with a distinct zona pellucida and extrusion of a polar body</td>
</tr>
<tr>
<td>Length of culture</td>
<td>6 days – 32 weeks</td>
<td>3 days – 24 weeks</td>
<td>4–24 hours</td>
<td>8 days – 7 months</td>
<td>21–24 days</td>
</tr>
<tr>
<td>Laboratory techniques</td>
<td>Complex, multiple techniques required</td>
<td>Complex, multiple techniques required</td>
<td>Simple, basic laboratory techniques required</td>
<td>Moderate, animal laboratory required</td>
<td>Complex, multiple techniques required</td>
</tr>
<tr>
<td>Autotransplantation needed</td>
<td>No</td>
<td>Yes, when aimed at a transplantable oocyte</td>
<td>Yes, when purging intact ovarian tissue</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Restoration of hormonal balance</td>
<td>No</td>
<td>Yes, in theory when designed as a transplantable artificial ovary with ovarian stromal cells</td>
<td>Yes, when purging intact ovarian tissue fragments for autotransplantation</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Offspring after animal studies</td>
<td>Yes</td>
<td>Yes</td>
<td>No animal testing reported</td>
<td>Yes</td>
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</tr>
</tbody>
</table>
know-how that is generally not present in fertility clinics.

The in-vivo development of an oocyte from a primordial follicle to the MII stage is assumed to take up to 8 months (Gougeon, 2010). In comparison, the maturation time during IVM is considerably shorter and the MII stage may already be reached after 21 days. Whether this rapid folliculogenesis affects oocyte quality is still unknown but after IVM abnormal MII oocytes with atypical large polar bodies were obtained (McLaughlin et al., 2018). In contrast, Xu and colleagues obtained MII oocytes with a normal-sized first polar body, meiotic spindle and typical ultrastructure after IVM (Xu et al., 2021). Due to these conflicting results, conclusions cannot yet be drawn about the effect of IVM on oocyte quality.

In mice, embryos and live offspring from IVM-derived oocytes have been described (Eppig et al., 2009; O’Brien et al., 2003). However, the pups showed a reduction in pulse rate and cardiac output, which may indicate that IVM has adverse effects on mice oocytes (Eppig et al., 2009). These results strongly indicate that additional testing is required to analyse the effects of IVM on human oocytes and the resulting children before IVM can be considered safe. IVM presents itself as a promising strategy but clearly requires further optimization before clinical application can be considered.

In terms of artificial ovaries there are two different approaches for the restoration of fertility (Figure 2). First, artificial ovaries could be used as an addition to IVM in providing physical support to growing follicles and maturing oocytes in vitro. Second, an artificial ovary can be designed for transplantation into the human body. Although several research groups have shown promising results, major challenges remain. One of the challenges of developing an artificial ovary is the changes in physical support that oocytes require during the various steps of maturation and the ability of the artificial ovary to co-evolve during this process. It should provide enough support for primordial follicles to maintain structural integrity, but it also needs to stretch or partially degenerate to provide enough room for follicle growth to the antral stage and ultimately ovulation (Holesh et al., 2021). In addition to these characteristics the artificial ovary should be sufficiently permeable for the diffusion of oxygen and nutrients essential not only for the survival of follicles during culture, but also for the process of neovascularization after transplantation.

Ideally, other ovarian cells, like ovarian stromal cells, are also seeded together with the follicles in the artificial ovary. The ovarian stromal cells have the capacity to recruit theca cells, which are required for steroid hormone production and growth factors essential for folliculogenesis (Chiti et al., 2017; Magoffin 2005). However, the introduction of ovarian cells from the cryopreserved ovarian tissue consequently results in the risk of introducing contaminating malignant cells into the artificial ovary, negating its purpose of safe fertility restoration. As an alternative, ovarian stromal cells could be harvested from the cured patient through a cortical biopsy, but so far these cells have only been obtained from the frozen-thawed ovarian cortex (Paulini et al., 2016).

When a transplantable artificial ovary is clinically applied, the material of which the ovary is constructed should meet the clinical standards required for transplantation. One of the problems relating to artificial ovaries is to determine which material is most suited for its construction, leading to optimal follicle survival and oocyte maturation. An artificial ovary from decellularized ovarian tissue rationally provides a matrix most closely resembling the in vivo situation (Pors et al., 2019). A close resemblance to the ultrastructure and rigidity of human ovarian cortex tissue is also reached through a F50/T50 matrix (Chiti et al., 2018). However, the low follicle recovery rates indicate that these matrices and culture conditions might not yet be fully optimized (Laranda et al., 2014; Pors et al., 2019; Yin et al., 2016).

Promising results have been reported in animal studies where offspring have been established in a mouse model, resulting in healthy pups after the transplantation of an artificial ovary (Gosden, 1990; Laranda et al., 2017). However, in studies with human ovarian tissue MII oocytes could only be obtained by the culture of small antral follicles in an artificial ovary without subsequent xenotransplantation (Kratz et al., 2010). All other studies reported an antral stage or even pre-antral stage as the end-point of the follicle development after culture in an artificial ovary. These studies were generally focusing more on the examination of follicular survival than on follicle growth. Before the clinical application of an artificial ovary can be considered, a more comprehensive understanding of the requirements of folliculogenesis is needed in order to design a supporting extracellular matrix that can mimic the in-vivo situation as closely as possible or even improve its function.

It is, of course, of prime importance to avoid the reintroduction of cancer cells. Follicles free from tumour cell contamination can be obtained by manually picking up follicles from ovarian tissue cell suspensions followed by multiple washes. These purified follicles are then suitable for IVM or can be implanted in an artificial ovary. However, purging is also described as an approach for the eradication of contaminating cancer cells from intact ovarian cortex tissue fragments prior to OTT by using tumour-cell specific inhibitors (Figure 2). Purging of intact tissue fragments is relatively straightforward and requires only minimal time, effort, technical skills and equipment. An additional major advantage is that pharmacological purging is the only experimental strategy so far that leaves the tissue architecture intact. Folliculogenesis depends on the microenvironment, and dissociation of the ovarian tissue, which is required for follicle isolation, is likely to have an impact on follicle development. By leaving the tissue fragment intact during purging the follicles remain in their natural microenvironment, promoting normal folliculogenesis, while ovarian hormonal activity is also more likely to be restored after OTT (Gellert et al., 2018).

An ideal pharmacological inhibitor for purging should meet a number of requirements, including having a low molecular weight to that it can obtain full tissue penetration during short-term culture to eliminate all contaminating malignant cells. A small-sized inhibitor is also likely to diffuse more efficiently out of the ovarian cortex fragment after purging to avoid any residual effect of the inhibitor on the patient after OTT. In addition, the inhibitor should have specific onco-suppressive characteristics that solely target the malignant cells without harming the ovarian cortex tissue or follicles. The choice of a
particular inhibitor for purging is not limited by possible systematic side effects of the agent. Even when an inhibitor cannot be used for clinical application in therapeutic doses, due to adverse systemic side effects, it is still possible to use it for purging ovarian tissue ex vivo as the woman’s other tissues will not be exposed to the drug. This is demonstrated by purging experiments using the Aurora kinase inhibitor GSK1070916, which causes neutropenia in human clinical trials but has proven to be highly effective in purging metastasized acute and chronic myeloid leukaemia from ovarian tissue (Eijkenboom et al., 2021). This provides, in theory, a wide range of different inhibitors for future purging strategies, including those that have been shown to have potent anti-cancer properties but limited clinical use because of unwanted systemic side effects.

Purging experiments directed at eliminating malignant cells from ovarian cortex fragments have shown that each malignancy may require a different inhibitor (Díaz-García et al., 2019; Mulder et al., 2019). This indicates that each malignancy may require a different purging regimen, and this appropriate purging method should first be identified for each individual malignancy to provide safe autotransplantation.

The advances in purging with intact ovarian cortex have opened up an additional and promising strategy for safe fertility restoration with cryopreserved ovarian tissue. This strategy leaves the follicle microenvironment intact and most closely resembles the current applied OTT while reducing the risk of malignant cell contamination. Nonetheless, the possible detrimental effects of the pharmacological inhibitors on follicles, oocytes and offspring in long-term animal studies have not yet been evaluated. In addition, the absence of malignant cells in purged ovarian tissue needs to be investigated after long-term xenografting before purging can be considered for clinical application.

Ovarian cortex tissue fragments xenografted to animals could serve as a source of mature oocytes suitable for IVF. This would circumvent the problem of reintroducing malignant cells since cancer cells are unable to pass the zona pellucida, therefore, after the recovery of mature oocytes from the graft, followed by IVF, the resulting embryos will be free of cancer cells (Dolmans and Amorim, 2019; Rios et al., 2018).

One of the major concerns of generating fertilizable oocytes with the aid of xenotransplantation is the risk of animal-borne pathogen transmission to the human population (Coazzi et al., 2005). This issue has become especially worrisome in view of the current COVID-19 pandemic that is possibly of zoonotic origin. In cases where mature oocytes from xenografts are used for IVF the risk of extracellular pathogen transmission is minimized by the fact it is only the oocyte and not the tissue fragment that will be retrieved from the graft. By using pathogen-free animals and regular testing the risk could be further reduced (Ekser et al., 2012).

In mice, the transplantation site of the ovarian graft seems to be less relevant as grafting to the kidney capsule, subcutaneous space or ovarian bursa or between the perimysium all lead to the formation of secondary or antral follicles. Although the spontaneous growth of follicles after xenografting has been described, indicating that mouse LH and FSH are able to stimulate the human ovarian tissue graft (Lotz et al., 2014a; Oktay et al., 2000), a higher yield of MII oocytes and antral follicles is to be expected after the administration of exogenous gonadotrophins to mice. A drawback of xenotransplantation is the low recovery rate of MII oocytes, resulting in a low number of fertilizable oocytes per graft, similar to IVM (Lotz et al., 2014a; Lotz et al., 2014b; Soleimani et al., 2010).

As with some of the other strategies using animals for study purposes, the xenotransplantation of ovarian tissue could give rise to ethical problems with, for example, the risk of pathogen transmission, the safety of the future mother and child, and animal rights. These ethical concerns inherent to xenotransplantation should be addressed prior to clinical application as a strategy to obtain embryos.

Xenotransplantation of ovarian tissue has shown that human follicle development and oocyte maturation is possible to MII stage in animals. Although these oocytes appear to have a normal morphology, more detailed analysis has revealed a clumpy meiotic spindle and impaired attachment of the chromatin to the metaphase plate (Kim et al., 2005). Further studies are required to determine whether these structural changes are caused by the freeze-thaw process of the ovarian tissue or by suboptimal conditions provided by the host animal. In addition other safety, legal and ethical issues should be resolved prior to the start of clinical experiments.

Relating to stem cell-based oogenesis, multiple studies have shown that VSEL and OGSC undergo meiosis in vitro and develop into oocyte-like structures (Bukovsky et al., 2005; Johnson et al., 2004; MacDonald et al., 2019; Porte et al., 2011; Silvestris et al., 2018; Virant-Klun et al., 2008; White et al., 2012). Because of these discoveries, the concept that women have a fixed reserve of oocytes seems to have been refuted, as these stem cells appear to have the potential to form mature oocytes later in life. Ideally, these stem cells isolated from ovarian tissue will provide mature autologous oocytes through in-vitro gametogenesis that are suitable for IVF without the need for autotransplantation of ovarian tissue. Stem cell-based oogenesis could potentially not only provide safe fertility restoration after OTC, but, possibly for other women with a diminished ovarian reserve, also provide renewed fertility by taking an ovarian cortical biopsy still harbouring these stem cells. Generating oocytes from VSEL could even be possible without taking an ovarian biopsy as these cells have also been identified in bone marrow and peripheral blood (Shin et al., 2013; Zuba-Surma et al., 2011). However, most studies on stem cell-based oogenesis focus on expanding the basic knowledge about the process of oogenesis and not on enhancing the safety of fertility restoration in cancer patients. Furthermore, most studies do not use human ovarian tissue as a source of stem cells and therefore these studies were not included in this systematic review.

Stem cell-based oogenesis still has a long way to go before clinical application can be considered. So far, the generation of fully mature oocytes and offspring from stem cells in vitro has only been accomplished in mice (Hikabe et al., 2016; Zou et al., 2009; Zhou et al., 2014). Human oogenesis starting from stem cells has not reached the stage beyond oocyte-like cells. These oocyte-like cells are morphologically similar to
ovocytes and express germ cell-specific markers at the mRNA and protein levels. However, oocyte-like cells have a lower gene expression level and do not express all of the important oocyte-specific genes, indicating that these cells are still not close to resembling viable oocytes (Virant-Klun et al., 2013). The presence of VSEL-like stem cells in borderline and high-grade serous carcinomas illustrates the possible carcinogenic properties of VSEL (Barabási et al., 2021; Virant-Klun and Stimpfel 2016; Virant-Klun et al., 2016). The potential of VSEL to form tumours under inappropriate conditions in the human body gives rise to serious concerns about the effects of those cells on the children derived from embryos from those stem cells, and on their mothers. The use of VSEL for oogenesis should thus be very carefully evaluated prior to implementing this technique in clinical experiments.

The processes and mechanisms that may lead to the formation of mature human oocytes from stem cells in ovarian tissue are still largely unknown. Further research is required before these cells could be used to generate oocytes for reproductive purposes. In-vitro stem cell-based oogenesis in humans remains a challenging and long-term goal, and even when this goal is reached it will still require IVM to obtain mature oocytes.

The strategies for the safe restoration of fertility highlighted in this review are all still in the experimental phase but show promising results for future clinical application. However, concerns regarding their effect on the resulting human offspring remain. These concerns should preferably be addressed by phenotypic and genetic analysis of the embryos after fertilization of the matured oocytes. The possibilities to fertilize human oocytes after applying these strategies are, however, severely limited due to legal and ethical restrictions. None of the strategies directed at increasing the safety of OTT of ovarian cortex tissue from cancer patients has yet been used in clinical practice and consequently the effects on the offspring remain unknown. Whether the treatment will result in epigenetic changes, a greater risk of disease or physical defects in human offspring can only be partially answered by animal studies. Although several studies using IVM, artificial ovaries, xenotransplantation or stem cell-based oogenesis have produced offspring in animals, clinical studies are inevitably needed to answer these questions for human offspring.

In addition to the possible effects on future children it is also important to be aware of possible effects on the mothers. If transplantation is being undertaken, laparoscopic or laparotomic surgery will be required, posing a surgical risk to the future mother, similar to the OTC procedure. Avoiding transplantation because of these surgical risks might be preferable, especially for women who have had previous abdominal surgery, with the possibility of adhesions (van Goor, 2007). Transplantation of an artificial ovary or purged ovarian cortex fragment to the pelvic area could also be unsuccessful because of a reduced revascularization of the graft caused by fibrosis after pelvic radiation during cancer treatment regimens (Dolmans et al., 2021). For these women, it is preferable to obtain fully mature and fertile oocytes in vitro. The transplantation of purged ovarian cortex fragments or an artificial ovary may also have additional beneficial effects such as a restoration of hormonal balance and the possibility of having multiple children after a single transplantation (Gellert et al., 2018).

However, adverse effects such as an increase in obstetric risks due to the in-vitro manipulation of ovarian tissue, oogenesis and folliculogenesis cannot be ruled out. For example, small amounts of anti-tumour drugs used during purging might persist in the tissue fragment and lead to systemic side-effects after grafting. Even procedures commonly used in assisted reproductive medicine are known to have a negative impact on obstetric and perinatal outcomes (Lei et al., 2019; Pondey et al., 2012). This stresses the importance of monitoring future mothers, and the analysis of gametes and embryos generated by these new techniques, aiming to increase the safety of fertility restoration. Obviously, the long-term follow-up of children born with using these procedures is also imperative.

The progression of the different techniques to clinical application will probably be accelerated by a more profound understanding of the complex processes involved in oogenesis, folliculogenesis and the role of the ovarian microenvironment. Scientific progress will bring new knowledge about these processes and will contribute to resolving the problems that are still associated with techniques for the safe restoration of fertility after gonadotoxic treatment for cancer.

**CONCLUSION**

This review highlights the great progress in research aimed at improving the safety of fertility restoration in oncological patients by OTT. Multiple strategies to avoid the problem of reintroducing cancer cells by OTT are being developed but substantial additional research is required before clinical application can be considered. Despite the fact that these experimental procedures are still in the experimental phase, they do offer hope for the future. It may take many years before an oncological patient will return for the autotransplantation of her ovarian tissue that her clinician preserves today. Hopefully, by then, some of these strategies will have reached the clinical stage and will provide such women with an effective and safe option to restore their fertility.

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