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Corresponding Author:	Sophie Brouillet, PhD, PharmD Universite de Montpellier Montpellier, FRANCE FRANCE
Corresponding Author's Institution:	Universite de Montpellier
Corresponding Author E-Mail:	s-brouillet@chu-montpellier.fr
Order of Authors:	Sophie Brouillet, PhD, PharmD
	Alice Ferriere-Hoa
	Alice Fournier
	Guillaume Martinez
	Julien Bessonnat
	Aurore Gueniffey
	Anna Gala
	Vanessa Loup
	Samir Hamamah
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TITLE:

Cryopreservation of Oocytes Retrieved from Ovarian Tissue to Optimize Fertility Preservation in Prepubertal Girls and Women

AUTHORS AND AFFILIATIONS:

Brouillet Sophie[1,2], Ferriere-Hoa Alice[2], Fournier Alice[2], Martinez Guillaume[3], Bessonnat Julien[3], Gueniffey Aurore[3], Gala Anna[2], Loup Vanessa[2], Hamamah Samir[1,2]

[1]Univ Montpellier, INSERM 1203, Développement Embryonnaire Précoce Humain et Pluripotence, Montpellier, France
[2]CHU Montpellier, Univ Montpellier, Département de Biologie de la Reproduction, Biologie de la Reproduction/DPI et CECOS, Montpellier, France
[3]CHU Grenoble-Alpes, Univ Grenoble-Alpes, Centre d'AMP-CECOS, Grenoble, France

Email addresses of co-authors:
Ferriere-Hoa Alice (a-ferrieres@chu-montpellier.fr)
Fournier Alice (alice-fournier@chu-montpellier.fr)
Martinez Guillaume (gmartinez@chu-grenoble.fr)
Bessonnat Julien (jbessonnat@chu-grenoble.fr)
Gueniffey Aurore (agueniffey@chu-grenoble.fr)
Gala Anna (a-gala@chu-montpellier.fr)
Loup Vanessa (v-loup@chu-montpellier.fr)
Hamamah Samir (s-hamamah@chu-montpellier.fr)

Corresponding author:
Brouillet Sophie (s-brouillet@chu-montpellier.fr)

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SUMMARY:
We propose a protocol for fertility preservation in prepubertal girls and women at risk of premature ovarian insufficiency. It combines ovarian tissue freezing and cryopreservation of oocytes retrieved from ovarian tissue. This strategy improves the safety and optimizes the reproductive potential of fertility preservation, maximizing the chance of childbirth.

ABSTRACT:
Human ovarian tissue cryopreservation (OTC) is increasingly used worldwide to preserve female fertility in prepubertal girls and women at risk of premature ovarian insufficiency (POI) in the context of urgent gonadotoxic treatments or ovarian surgery. Fertility preservation is challenging because there is no consensus regarding patient management, preservation fertility strategies, or even technical laboratory protocols, which implies that each procedure must be adapted to the characteristics of the patient profile and its own risk-benefit ratio. During OTC, mature/immature oocytes can be aspirated directly from large/small antral follicles within ovarian tissue samples and/or be released into culture media from growing

follicles during ovarian tissue dissection in prepubertal girls and women. In this manuscript, we present a protocol that combines ovarian tissue freezing with the cryopreservation of mature/immature oocytes retrieved from ovarian tissue samples, improving the reproductive potential of fertility preservation. Appropriate collection, handling, and storage of ovarian tissue and oocytes before, during, and after the cryopreservation will be described. The subsequent use and safety of cryopreserved/thawed ovarian tissue samples and oocytes will also be discussed, as well as the optimal timing for in vitro maturation of immature oocytes. We recommend the systematic use of this protocol in fertility preservation of prepubertal girls and women as it increases the whole reproductive potential of fertility preservation (i.e., oocyte vitrification in addition of OTC) and also improves the safety and use of fertility preservation (i.e., thawing of oocytes versus ovarian graft), maximizing the chance of successful childbirth for the patients at risk of POI.

INTRODUCTION:

The field of fertility preservation has grown over the last two decades due to the increasing number of patients at risk of premature ovarian insufficiency (POI)[1–3]. The current available medical options to preserve fertility are ovarian tissue cryopreservation (OTC)[4], oocyte/embryo freezing after ovarian stimulation[5], administration of GnRH analogues[6], or ovarian transposition[7]. OTC is a major advance for fertility preservation, particularly in prepubertal girls, where it is the only option currently available to preserve fertility and also in women who cannot delay the onset of their gonadotoxic treatment[2,4].

OTC allows the preservation of a high number of primordial follicles, which are located in the outer 1 mm of the ovarian cortex[2]. Frozen/thawed ovarian tissue can be subsequently used by graft (orthotopic or heterotopic, autologous or donor) or cultured in vitro to obtain mature oocytes[2]. The graft of frozen-thawed prepubertal ovarian tissue samples has been shown to induce puberty[8,9]. In women, the reproductive outcomes after orthotopic autografting of frozen-thawed ovarian cortex are reassuring, with live birth rates reaching 57.5% after natural conceptions[10] and between 30% to 70% after Assisted Reproductive Techniques (ART) conceptions[11]. Since the first live birth from orthotopic transplantation of frozen/thawed human ovarian tissue in 2004[12], this technique allowed the birth of at least 130 children worldwide[2]. The hormonal and reproductive functions of the graft tissue generally last for several years[11,13,14], confirming its long-term functionality.

However, auto-transplantation of ovarian tissue samples carries a theoretical risk of reintroducing viable malignant cells in some patients[15–18], particularly in leukemia survivors[19]. To date, no case of transmission of cancer via the graft of frozen/thawed ovarian cortex has been reported in healthy cancer survivors[11], suggesting that the fibrous avascular nature of the ovarian cortex could represent an inhospitable microenvironment for the dissemination of malignant cells. Nevertheless, the graft of ovarian tissue still represents an experimental and challenging technique, indicating that the use of oocytes should be currently considered as an easier and safer approach than ovarian tissue graft to restore fertility. Interestingly, immature oocytes could be easily retrieved from ovarian tissue during OTC in both prepubertal girls and women[16], suggesting that it could represent a reliable source to maximize the fertility-restoring potential in addition to ovarian cortical tissue freezing. These oocytes could be aspirated manually ex vivo in the ART laboratory from visible antral follicles or be isolated from spent media after ovarian tissue dissection. Retrieved oocytes could then

be directly vitrified at an immature stage or be matured before the vitrification step using in vitro maturation (IVM)[20,21].

In this manuscript, we propose a protocol that combines the cryopreservation of ovarian tissue with the isolation and cryopreservation of mature (MII-stage oocytes) and/or immature oocytes (i.e., Germinal Vesicle (GV) and Metaphase I (MI)-stage oocytes) retrieved from ovarian tissue. This protocol outlines all the specific steps required to maximize the fertility preservation potential in both prepubertal girls and women.

PROTOCOL:

All women (over 18 years of age) as well as all minor girls and their parents signed an informed consent form to preserve the fertility of the patient at risk of POI. This protocol is considered as a routine ART procedure in our center for fertility preservation. It follows the guidelines of our institutions human research ethics committee.

1. Quality control

1.1 Include female age ≤ 38 years, containing circulating anti-mullerian hormone concentration >1 ng/mL and showing the presence of an increased risk of POI due to gonadotoxic treatment. Exclude women eligible for oocyte vitrification for fertility preservation procedure, women with a condition that prevents giving a fully informed consent or women with a high risk of complications from anesthesia or surgery.

1.2 Confirm the presence of a signed patient consent before initiating this procedure.

1.3 Identify two vacant locations within two distinct cryopreservation storage tanks. Half of the cryotubes/straws will be kept in the first storage tank while the other half of the cryotubes/straws will be kept in the other tank to minimize the risk of a total loss of the samples in the case of a tank failure.

1.4 Check the availability and functionality of all medical devices required in this protocol. Sterile surgical dissecting scissors with sharp straight blades and finely sharpened points as well as sterile atraumatic tissue forceps are recommended for the dissection step.

1.5 Check that all quality-control steps have been respected for all medical devices (particularly, validation and routine control of sterilization processes, expiration/lot number verification and tracking).

1.6 Maintain an aseptic/a sterile environment throughout the procedure and realize with caution all the safety precautions.

1.7 Use different dishes, pipettes, and surgical instruments for each patient.

2 The day before the preservation

2.1 Prepare one sterile 60 mm IVF Petri dish containing an IVF medium covered with mineral oil and incubate the dish overnight at 37 °C in an atmosphere of 5% O_2 and 5% CO_2.

Use this dish to collect the cumulus-oocyte complexes (COCs) that will be potentially retrieved from the tissue (see section 7.2).

2.2 Prepare one sterile 35 mm IVF Petri dish containing IVF medium and incubate the dish overnight at 37 °C in an atmosphere of 5% O_2 and 5% CO_2. Use this dish for the oocyte denudation step (see section 9.1).

2.3 Prepare one sterile 35 mm IVF Petri dish containing IVF medium covered with mineral oil and incubate the dish overnight at 37 °C in an atmosphere of 5% O_2 and 5% CO_2. Use this dish to collect the oocytes after the denudation step (see section 9.4).

3 Ovarian tissue collection and transport

3.1 Perform the laparoscopic surgery under general anesthesia using one 10 mm port positioned at the umbilicus and two 5 mm ports, one positioned at the left-lower quadrant and another one positioned at the right.

3.2 Perform a partial or complete unilateral oophorectomy according to the consensus decision of the oncologist, the surgeon and the medical staff of the ART unit based on the ovarian reserve, the size, and the macroscopic aspect of both ovaries (**Figure 1, A1–B1**) and the planned gonadotoxic protocol. Perform oophorectomy with sharp scissors or a surgical staple. Do not use any dissection device that could induce collateral, electric, or thermal injury to the ovarian tissue that is to be preserved.

NOTE: A complete unilateral oophorectomy is generally performed in prepubertal girls[16,22], whereas a partial unilateral oophorectomy could be commonly achieved in adult patients with large ovaries and/or a high ovarian reserve.

[Place **Figure 1** here]

3.3 Perform the evacuation of the ovarian tissue using a handmade or commercial laparoscopic specimen retrieval bag.

3.4 Put the ovarian tissue in a sterile tube containing culture medium at 4 °C. The tissue must be completely immersed in the culture medium.

3.5 Immediately transport the ovarian tissue in an insulated pouch at 4 °C to the ART laboratory for optimal results.

NOTE: If necessary, the ovarian tissue can be transport at cold temperatures (around 4 °C) to the laboratory for up to 26 h after the collection without threatening the quality of the tissue[23].

4 Ovarian tissue preparation

4.1 Transfer the ovarian tissue into a sterile 90 mm IVF Petri dish containing 20 mL of pre-cooled culture medium at 4 °C.

4.2 Put the Petri dish containing the ovarian tissue on a cold plate (at 4 °C) placed on a vertical laminar flow clean bench to minimize the risk of microorganism contamination.

5 Manual follicle aspiration from ovarian tissue samples

5.1 Aspirate visible antral follicles (if present on the surface of ovarian tissue) with a 21 G syringe needle connected to a 1 mL disposable syringe.

NOTE: This step could be tricky. An alternative may be to gently open each visible antral follicle with a scalpel and rinse the inside of each follicle with IVF media in order to softly release COCs within the dissection media.

5.2 Flush the collected follicular fluids into a sterile 60 mm IVF Petri dish containing 5 mL of IVF culture medium at room temperature.

5.3 Rinse the syringe with 1 mL of IVF culture medium at room temperature and flush the liquid into the same IVF Petri dish. Repeat this step two times.

5.4 Identify and isolate the COCs under an inverted microscope (50x–200x magnification).

5.5 Transfer the healthy COCs with a pipette into a new sterile 60 mm IVF Petri dish containing pre-equilibrated IVF medium covered with oil at 37 °C in an atmosphere of 5% O_2 and 5% CO_2.

NOTE: Healthy COCs contain a translucent oocyte. Discard the atretic COCs presenting a brown coloured oocyte.

5.6 Store the IVF Petri dish in the incubator at 37 °C in an atmosphere of 5% O_2 and 5% CO_2 until the denudation step (see step 9).

6 Ovarian tissue dissection

6.1 Prepare the fresh freezing solution in a 50 mL sterile tube. The freezing solution contains 1.5M dimethylsulfoxide (DMSO) and 10% of human serum albumin in IVF culture medium. Gently vortex.

NOTE: It should be prepared just before use.

6.2 Cut the ovarian cortex into slices measuring 10 mm x 10 mm x 1 mm or 0.5 mm x 0.5 mm x 1 mm in the case of complete or partial unilateral oophorectomy, respectively (**Figure 1,A2–B2–B3**). The cutting is performed with sterile surgical sharp scissors and atraumatic forceps.

6.3 Remove medulla as much as possible (**Figure 1, B4**).

NOTE: One slice of ovarian tissue is kept intact with both ovarian cortex and medulla for

236 further histological analysis (see step 8).
237
238 6.4 After the dissection, perform two washing steps (or more, if required) for each ovarian
239 cortical sample in 1 mL of IVF culture medium to remove blood. After the washing, transfer all
240 the tissue samples into a new 60 mm Petri dish with fresh IVF culture medium.
241
242 6.5 Place each ovarian cortical sample in a cryotube containing 1 mL of freezing solution.
243
244 NOTE: Mixing is unnecessary. The slice of ovarian tissue kept for further histological analysis
245 is not cryopreserved (see step 8).
246
247 6.6 Keep the samples for 30 min at 4 °C for equilibration with the freezing solution.
248
249 6.7 Cryopreserve all samples with a slow freezing technique. The cooling rate is -2 °C/min
250 from +4 °C until -9 °C, -50 °C/min until -30 °C; hold during 1 min, +4 °C/min until -15 °C, -2
251 °C/min until -40 °C; and finally -25 °C/min until -150 °C using a programmable freezer.
252
253 6.8 Remove the cryotubes immediately and plunge them into liquid nitrogen at -196 °C.
254
255 6.9 Place half of the cryotubes in a first liquid nitrogen storage tank and the other half of
256 the cryotubes in a second liquid nitrogen tank.
257
258 **7 Manual COC isolation from the dissection spent media**
259
260 7.1 Identify COCs from the dissection spent media under a microscope (50x–200x
261 magnification) (**Figure 2**).
262
263 [Place **Figure 2** here]
264
265 7.2 Transfer the healthy COCs with a pipette into a new sterile 60 mm IVF Petri Dish
266 containing pre-equilibrated IVF medium covered with oil at 37°C in an atmosphere of 5% O_2
267 and 5% CO_2 (see step 2.1).
268
269 NOTE: Discard the atretic COCs.
270
271 7.3 Store the IVF Petri dish in the incubator at 37 °C in an atmosphere of 5% O_2 and 5%
272 CO_2 until the denudation step (see step 9).
273
274 **8 Ovarian tissue histological analysis**
275
276 8.1 Fix the remaining slice of ovarian tissue (containing both ovarian cortex and medulla)
277 in a 3% formalin solution in a chemical clean bench for histological examination.
278
279 NOTE: It should be performed only once the cortical samples are successfully frozen and
280 stored.
281
282 CAUTION: The formalin solution is irritating, corrosive, and toxic. This step should be carried

out carefully and outside the IVF laboratory.

8.2 Prescribe a complete histological analysis of this ovarian tissue sample with an evaluation of the potential presence of malignant cells as well as a description of the number and type of ovarian follicles (i.e., primordial, primary, secondary, and antral follicles, respectively).

9 Oocyte denudation and selection

9.1 Perform oocyte denudation. Briefly expose COCs to hyaluronidase solution (80 IU/mL or final concentration 0.1 mg/mL) with gentle pipetting (repeated aspirating and expelling the COCs into the denudation solution) using a large diameter pipette tip (volume 0.1–20 µL, length: 40.5 mm, diameter work cone: 6 mm, diameter opening: 0.36 mm) for 30 s, immediately followed by two washing steps in pre-equilibrated medium covered with mineral oil to remove the excess enzyme (see step 2.1).

9.2 Optimize the removal of cumulus and corona radiata cells by gentle pipetting using a 150 µm pipette tip in pre-equilibrated IVF medium (see step 2.1).

9.3 Perform a selection step of healthy oocytes using an inverted microscope (200x–400x magnification). The maturation stage of healthy oocytes could be GV, MI, or MII-stage oocytes (Figure 3). Healthy oocytes have the following morphological characteristics: an intact and round cytoplasm, a size between 100–150 µm, and a pale color.

NOTE: Discard atretic and unhealthy oocytes (**Figure 3,D1–D3**).

[Place **Figure 3** here]

9.4 Incubate healthy oocytes inside the incubator at 37 °C, 5% O_2 and 5% CO_2 until the vitrification step (see step 2.1).

10 Oocyte vitrification

10.1 Confirm patient/specimen identification.

10.2 Perform a new selection step of healthy oocytes using an inverted microscope (200x–00x magnification).

NOTE: Discard atretic and unhealthy oocytes.

10.3 Take a picture of each healthy oocyte using an inverted microscope just before the vitrification step. Note the maturation stage of each oocyte.

NOTE: Attach all these data (image, size, maturation stage) in the file of the patient.

10.4 Use different identification numbers per straw to differentiate them if cryopreserving more than one straw per patient.

10.5 Cryopreserve one or two oocyte(s) per straw.

NOTE: Mature and immature viable oocytes must be cryopreserved separately. When cryopreserving two oocytes together in the same straw, it is recommended to select oocytes with similar morphological characteristics.

10.6 Cryopreserve healthy oocytes using a vitrification kit according to the manufacturer's instructions (see **Table of Materials**).

10.7 Load the cryopreserve oocytes on an appropriate device for cryostorage in liquid nitrogen according to the manufacturer's instructions (see **Table of Materials**).

10.8 Place half of the straws in a first liquid nitrogen storage tank and the other half of the straws in a second liquid nitrogen tank.

REPRESENTATIVE RESULTS:

A total of 81 OTC from 81 female patients have been performed between 2007 and 2020, including 43 prepubertal girls and 38 women. The mean age of female patients (prepubertal girls and women) was 14.21 ± 9.61 years (mean ± standard error). The youngest patient was 5 months old and the oldest was 33.6 years old (**Table 1**). The mean age of prepubertal girls and women was 6.84 ± 4.81 years old (min–max: 0.5–15.1) and 22.76 ± 5.92 years old (min–max: 14.2–33.6), respectively (Mann-Whitney, $p = 3.37 \times 10^{-10}$).

The percentage of patients with a history of gonadotoxic treatment before OTC was 48.84% (21/43) in prepubertal girls and 13.16% (5/38) in women (Chi^2, p = 0.0006). A complete oophorectomy was performed for the majority of patients (88.89% (72/81), 43 prepubertal girls and 29 women) whereas a partial oophorectomy was only performed in 9 women (Fisher's Exact Test, p = 0.0006). **Figure 4** shows the negative impact of using a dissection device that could induce collateral electric or thermal injury to the ovarian tissue during oophorectomy. **Figure 5** shows the negative impacts of two different treatments on the quality of the collected ovarian tissue. The percentage of patients with a positive retrieval of oocytes from ovarian tissue was 41.86% (18/43) in prepubertal girls and 71.05% (27/38) in women (Chi^2, p = 0.0008). No oocytes could be isolated in 36 patients. The youngest patient from whom oocytes were retrieved was 3 years old whereas the oldest was 33.6 years old. The total number of mature and immature oocytes was 71 oocytes (min–max, 1–9 per patient) in prepubertal girls and 377 oocytes (min–max, 1–27 per patient) in women (Fisher's Exact Test, p = 1). The mean number of retrieved oocytes per oophorectomy was 1.65 ± 2.68 in prepubertal girls and 9.92 ± 9.51 in women (Mann Whitney, $p = 9.15 \times 10^{-5}$). In women, the mean number of retrieved oocytes was similar in partial oophorectomy compared to complete oophorectomy (13.11 ± 7.18 versus 8.93 ± 10.03, Mann-Whitney, p = 0.16). The mean number of retrieved oocytes per patient with a positive retrieval was significantly higher in women compared to prepubertal girls (13.98 ± 6.38 versus 3.94 ± 2.86, Mann-Whitney, p = 0.0003). The retrieval of mature oocytes (i.e., MII-stage oocyte) was extremely rare in women (i.e., only one mature oocyte from one woman) and inexistent in prepubertal girls. A total of 71 immature oocytes (i.e., 4 MI-stage oocytes and 67 GV-stage oocytes) were obtained from prepubertal girls and 376 immature oocytes (i.e., 28 MI-stage and 348 GV-stage oocytes) were

retrieved from women (Fisher's Exact Test, p = 0.8). The percentage of atretic oocytes was significantly higher in prepubertal girls compared to women (Chi^2, p = 1.43 x 10^{-7}).

The percentage of patients with a cryopreservation of oocytes retrieved from ovarian tissue was 23.26% (10/43) in prepubertal girls and 65.79% (25/38) in women (Chi^2, p = 0.0001) (**Table 2**). The total number of cryopreserved mature and immature oocytes was 38 oocytes (i.e., 2 MI-stage and 36 GV-stage oocytes) in prepubertal girls and 310 oocytes (i.e., 23 MI and 286 GV) in women (Fisher's Exact Test, p = 1 and p = 1, respectively). The mean number of cryopreserved oocytes per patient who had the chance to benefit from oocyte freezing was 3.80 ± 2.35 in prepubertal girls and 12.40 ± 6.40 in women (Mann-Whitney, p = 0.0008).

After fertility preservation, the majority of patients chose to continue the storage of ovarian tissue and oocyte samples (**Supplementary Table 1**). The main reason for fertility preservation destruction was patient death (9.30% (4/43) in prepubertal girls and 15.79% (6/38) in women). No request for use has so far been requested. The loss of follow up was 4.65% (2/43) in prepubertal girls and 10.53% (4/38) in women.

FIGURE AND TABLE LEGENDS:

Figure 1: Ovarian tissue cryopreservation. Ovarian tissue cryopreservation in a prepubertal girl (**A**, 7-year-old patient) and in a woman (**B**, 28-year-old patient) both suffering from acute myeloid leukemia. (**A1–B1**) Laparoscopic view of the ovary. (**A2–B2–B3**) Dissection of ovarian tissue. (**B4**) Cortical ovarian tissue. CL: corpus luteum. SAF: small antral follicle.

Figure 2: Mature and immature COCs retrieved from ovarian tissue. Mature (**A**) and immature (**B1–B3,C1–C2**) COCs retrieved from ovarian tissue. (**A**) Mature COC containing a mature oocyte (presence of the first polar body, Metaphase II-stage oocyte). (**B1**) Healthy immature COC containing an immature oocyte (no polar body, Metaphase I-stage oocyte). (**B2–B3**) Healthy immature COCs containing immature Germinal Vesicle-stage oocytes (no polar body, Prophase I-stage oocytes). (**C1–C2**) Unhealthy COCs containing an atretic brown colored oocyte.

Figure 3: Mature and Immature oocytes retrieved from ovarian tissue. (A) Mature oocyte (Metaphase II-stage oocyte: presence of the first polar body (PB, arrow), no visible nucleus), (**B1–B2**) Immature oocyte (Metaphase I-stage oocyte: no polar body, no visible nucleus), (**C1–C3**) Immature oocytes (Germinal Vesicle-stage (VG) oocytes: no polar body, presence of a large halo with nucleoli within the cytoplasm (arrow)). In **C1**, Germinal Vesicle-stage oocytes display heterogenous size. (**D1–D3**) Atretic oocytes. PB: polar body. GV: Germinal Vesicle.

Table 1: Patient clinical characteristics and biological parameters of oocyte retrieval from ovarian tissue. SE = standard error. [a]Statistical analysis of the number of oocytes retrieved from ovarian tissue obtained after partial oophorectomy compared to complete oophorectomy in women.

Figure 4: Ovarian tissue quality. Collected ovarian tissue of high quality (**A1–A2**) versus low quality (**B1–B2**). The low quality is due to the use of an inappropriate dissection device that had induced collateral injury to the ovarian tissue during oophorectomy.

Figure 5: Low quality ovarian tissue. Collected ovarian tissue of low quality due to the negative impacts of (**A**) ovarian puncture after hormonal stimulation performed 2 days before OTC and (**B**) recent chemotherapy treatment administrated the week before OTC.

Table 2: Proportion of patients who beneficiated from oocytes cryopreservation retrieved from ovarian tissue and biological parameters of cryopreserved oocytes.

Supplementary Table 1: Storage/destruction of fertility preservation and follow-up of patients. Fertility preservation: cryopreserved ovarian tissue +/- retrieved oocytes from ovarian tissue.

DISCUSSION:

The current manuscript provides a protocol combining ovarian tissue freezing and cryopreservation of oocytes retrieved from ovarian tissue, increasing the potential of fertility in prepubertal girls and women at risk of POI. We strongly recommend performing this protocol before the start of any gonadotoxic therapy in order to optimize the quantity (i.e., number of viable oocytes) as well as the quality (i.e., DNA integrity and cytoplasm competence) of the preserved oocytes, optimizing their safety for clinical use[24]. If it is not possible to perform this protocol before any form of gonadotoxic treatment, this protocol can also be conducted after the start of gonadotoxic therapy but an extreme caution is then advised when using these oocytes in ART, warranting specific precautions such as pre-implantation genetic testing (PGT), careful fetal monitoring, and amniocentesis[16].

In the absence of a clear consensus on the amount of ovarian tissue that should be retrieved for fertility preservation, we recommend to perform complete oophorectomy in prepubertal girls as well as in patients associated with a moderate/high risk of POI or with a low ovarian reserve. Indeed, the volume of ovarian tissue collected during oophorectomy will be directly correlated to the number of oocytes that can be retrieved and then cryopreserved for the same patient. In our results, we observed that the numbers of retrieved oocytes were similar in partial oophorectomy compared to complete oophorectomy in women, but this result reflects the differences in ovarian reserve between the patients to whom we proposed partial oophorectomy (i.e., young patients associated with a high ovarian reserve and a low risk of POI) and those to whom we proposed total oophorectomy (i.e., older patients associated with a low ovarian reserve and a moderate/high risk of POI)[16]. To date, no significant harm of unilateral oophorectomy on long-term female fertility has been reported and the onset of menopause seems similar (or at worst perhaps 1 year earlier in human), probably due to a compensatory mechanism that lead to slower recruitment rate of primordial follicles[11,25]. Hence, there seems to be no significant negative effect of complete oophorectomy while it could allow the cryopreservation of a high number of oocytes, increasing the chances of having a live birth. Moreover, the use of cryopreserved oocytes by in vitro fertilization is much easier and safer than the graft of cryopreserved/thawed ovarian cortical tissue because of the risk of reseeding the disease of the patient is then avoided. Hence, this approach is particularly appropriate in blood or metastatic diseases associated with a risk of possible malignancy reseeding from transplanted ovarian tissue[15–18], specifically in patients with leukemia[19].

To date, slow freezing seems to give better results than vitrification for the cryopreservation of ovarian tissue samples but vitrification is clearly better for oocyte freezing[26]. These results

explain why we choose to preserve the ovarian tissue with the slow freezing technique whereas retrieved oocytes were vitrified. We also choose to vitrify immature oocytes before any in vitro maturation (IVM) step because we hope that the performance of IVM will be significantly improved in future. At present, data is scarce on the optimal timing for oocyte vitrification (i.e., before or after IVM), preventing any conclusion on the most efficient strategy. Only one meta-analysis on this topic has been published in 2018[27], but it mainly concerns immature oocytes recovered after hormonal stimulation (and not oocytes retrieved from ovarian tissue) as well as it only evaluates oocyte maturation rate and no other clinical outcomes (such as fertilization rates, embryo development, or pregnancy/live birth rates). Moreover, the same IVM protocol was performed before and after the vitrification/thawing steps, excluding the impact of future advances in IVM protocols. As frozen oocytes for fertility preservation will not be used for several years or even decades in the case of prepubertal girls, progress will most certainly be made in IVM protocols in the coming years, giving hope for better results in terms of oocyte maturation rate as well as developmental/implantation potential.

Limited data is available on the developmental potential of oocytes derived from ovarian tissue after oophorectomy, specifically from prepubertal girls. Some studies reporting higher rates of atresia and abnormal morphology combined with a lower maturation potential[16,28,29] in oocytes retrieved from prepubertal girls compared to women, whereas other publications describing similar characteristics are reference[16,24,30–35]. To the best of our knowledge, there is no report of pregnancy after IVM of oocytes frozen during childhood or adolescence. In women, clinical pregnancy rates and neonatal morbidity seem similar after IVM compared to standard IVF/ICSI[36]. Further reports are warranted on the fertility-restoring potential of oocytes retrieved from ovarian tissue, specifically in prepubertal patients.

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DISCLOSURES:
None of the authors have competing interests.

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597

Figure 1

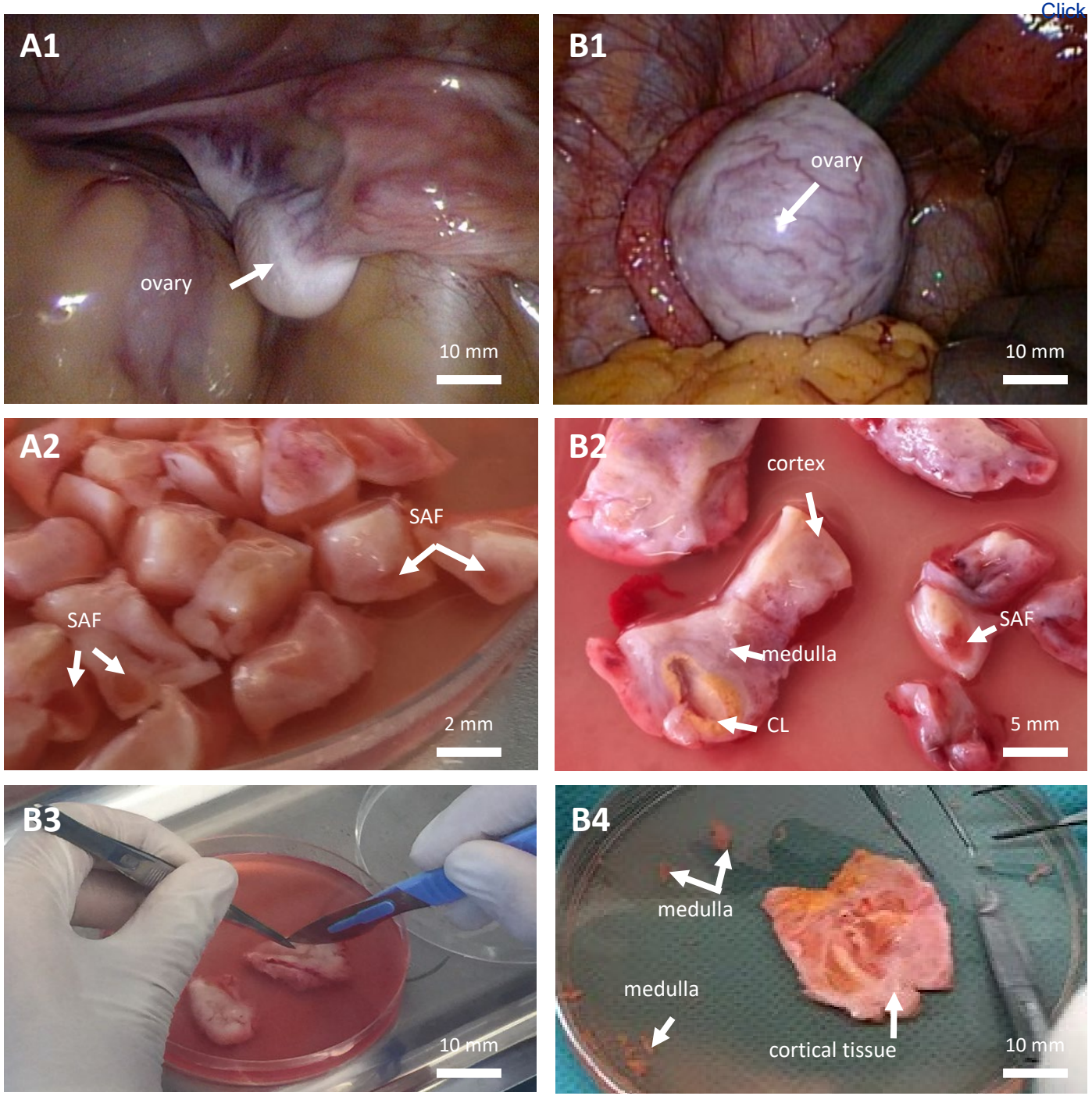

Figure 2

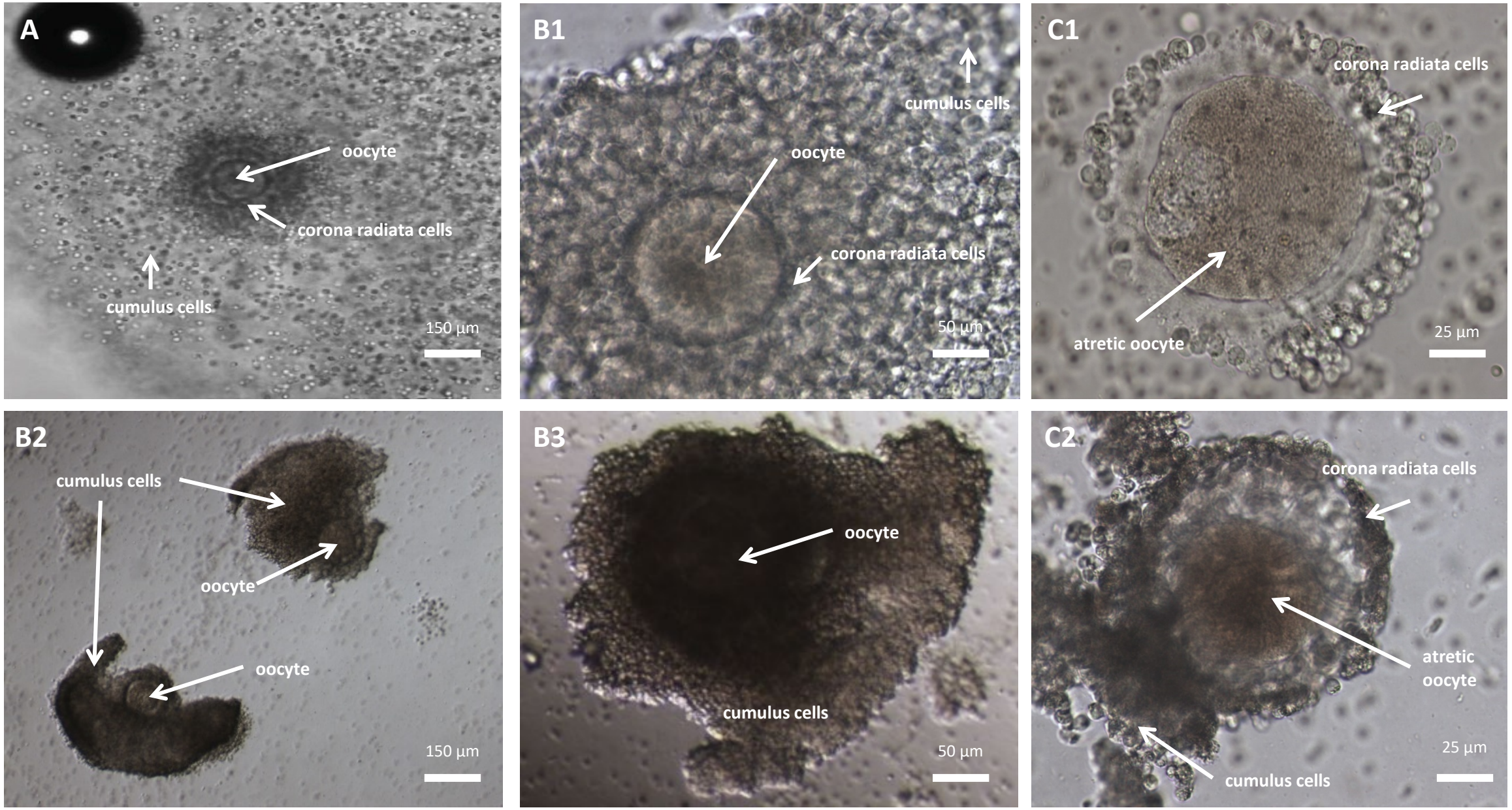

Figure 3

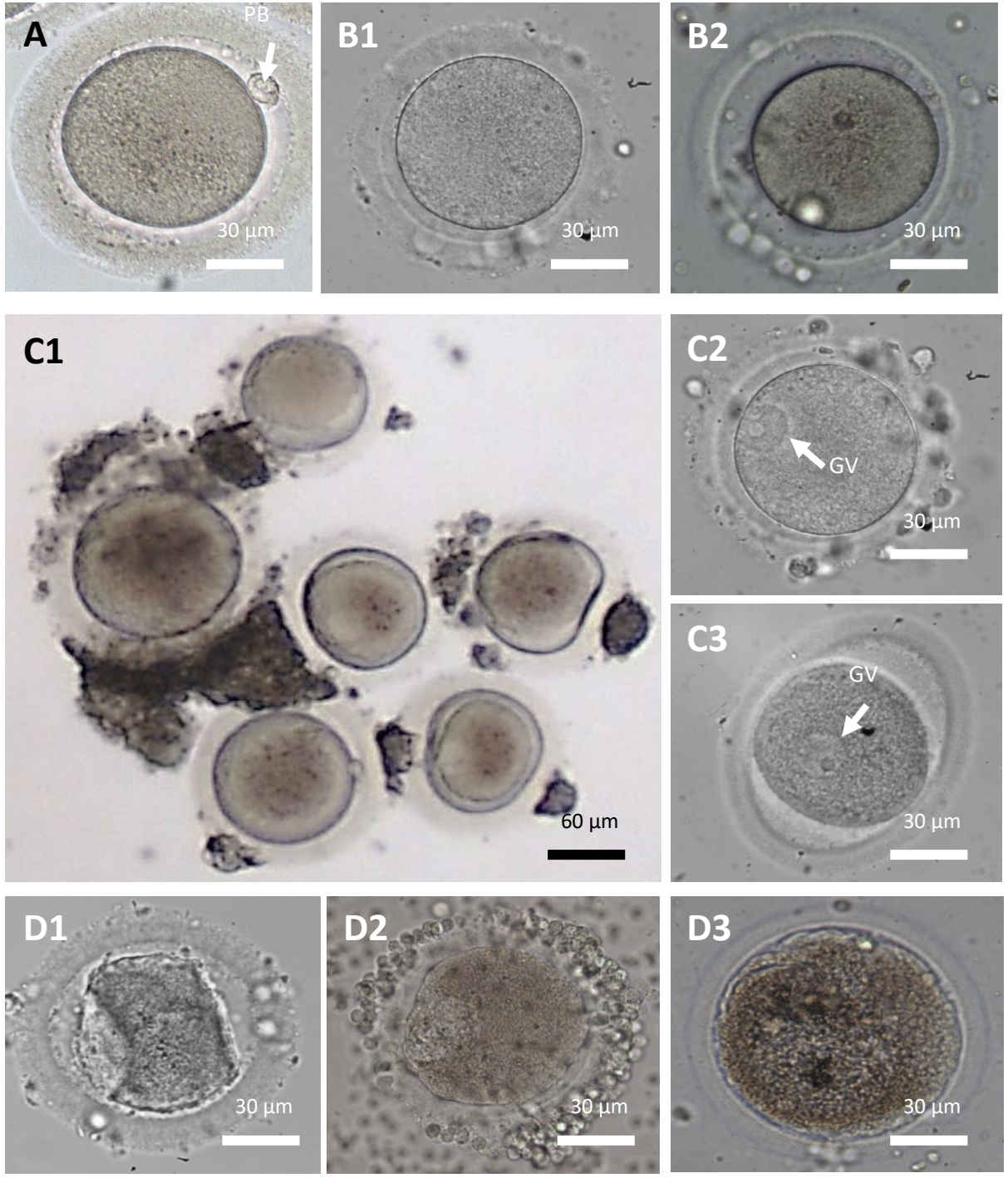

Figure 4

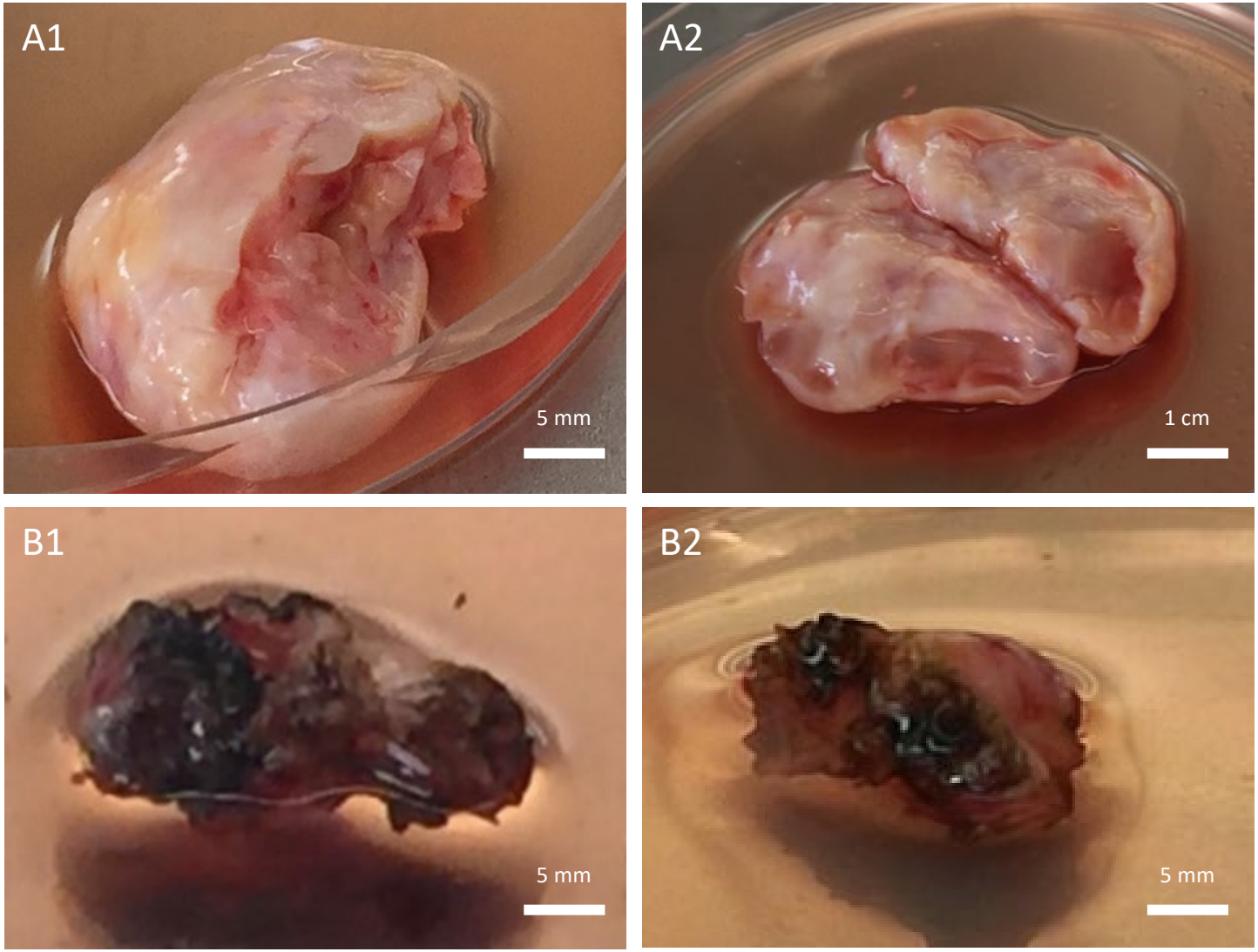

Figure 5

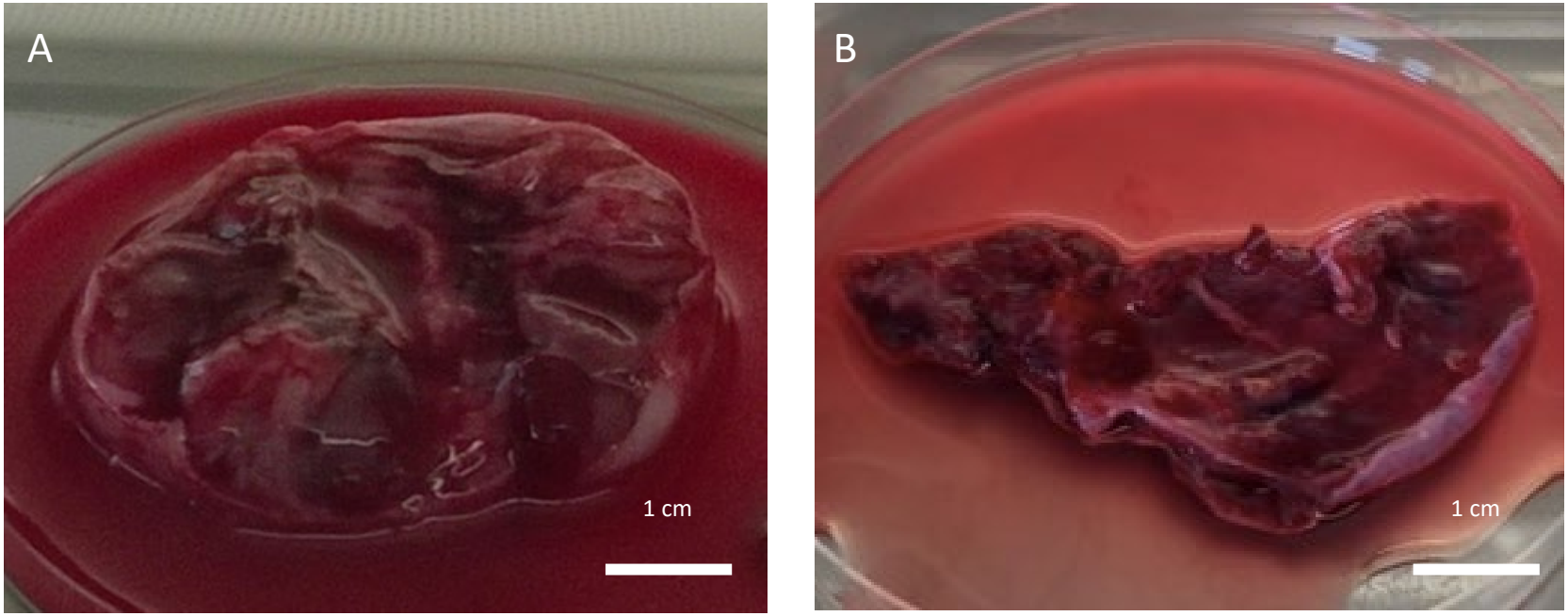

Table 1

	Prepubertal girls n=43	Women n=38	Statistical analyses	P value
Age (mean ± SE) (min-max)	6.84 ± 4.81 (0.5-15.1)	22.76 ± 5.92 (14.2-33.6)	Mann-Whitney	3.37×10^{-10}
% of patients with an history of gonadotoxic treatment	48.84% (21/43)	13.16% (5/38)	Chi^2	0.0006
Complete/partial oophorectomy	43/0	29/9	Fisher's Exact Test	0.0006
Number of retrieved oocytes per oophorectomy (mean +/- SE)	1.65 ± 2.68	9.92 ± 9.51	Mann-Whitney	9.15×10^{-5}
Number of retrieved oocytes per complete oophorectomy (mean +/- SE)	1.65 ± 2.68	8.93 ± 10.03[a]	Mann-Whitney	0.0068
Number of retrieved oocytes per partial oophorectomy (mean +/- SE)	-	13.11 ± 7.18[a]	Mann-Whitney	0.16[a]
% of patients with a positive retrieval of oocytes from ovarian tissue	41.86% (18/43)	71.05% (27/38)	Chi^2	0.008
Number of retrieved oocytes per patient with a positive retrieval (mean +/- SE)	3.94 ± 2.86	13.98 ± 6.38	Mann-Whitney	0.0003
Total number of mature oocytes (min-max per patient with a positive retrieval)	0 (0-0)	1 (0-1)	Fisher's Exact Test	1
Total number of immature oocytes (min-max per patient with a positive retrieval)	71 (1-9)	376 (1-26)		
Maturation stage of immature oocytes	4 M1	28 M1	Fisher's Exact Test	0.80
	67 GV	348 GV		
% of atretic mature and immature oocytes	46.48% (33/71)	18.04% (68/377)	Chi^2	1.43×10^{-7}

Table 2

	Prepubertal girls n=43	Women n=38	Statistical analyses	P value
% of patients with a cryopreservation of oocytes retrieved from ovarian tissue	23.26% (10/43)	65.79% (25/38)	Chi^2	0.0001
Number of cryopreserved oocytes per patient with a cryopreservation (mean +/- SE)	3.80 ± 2.35	12.40 ± 6.40	Mann-Whitney	0.0008
Total number of healthy cryopreserved mature oocytes (min-max per patient)	0 (0-0)	1 (0-1)	Fisher's Exact Test	1
Total number of healthy immature cryopreserved oocytes	38 (1-9)	309 (1-23)		
Maturation stage of healthy immature cryopreserved oocytes	2 M1	23 M1	Fisher's Exact Test	1
	36 GV	286 GV		

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 ml disposable syringe	CDD	1323101/7002655	Other material and sizes may also be suitable
21-gauge syringe needle	Merck	Z192481	Other material and sizes may also be suitable
35 mm IVF Petri Dish	Nunc	150255	Other material and sizes may also be suitable
60 mm IVF Petri Dish	Nunc	150270	Other material and sizes may also be suitable
90 mm IVF Petri Dish	Nunc	150360	Other material and sizes may also be suitable
Atraumatic forceps	Medlane	PI 299 04	Other material and sizes may also be suitable
Continuous Single Culture Complete with HSA	Irvine Scientific	90165	IVF culture medium for follicular fluid collection, COCs incubation, oocyte denudation and oocyte incubation until the vitrification step.
Cryotube	Thermo Scientific	368632	Other products may also be suitable
Dimethylsulfoxide (DMSO)	MILTENYI BIOTEC SAS	170-076-303	CryoMACS DMSO 10 (EP)
GT40	Air Liquide	113,517	Storage tank
HSV High Security Vitrification Straw	Irvine Scientific	25251	Vitrification straws
Human serum albumin	Vitrolife	10064	Other products may also be suitable
Leibovitz L15 medium	Eurobio	CM1L15000U	Culture medium for ovarian tissue collection, transport and tissue dissection
Leibovitz L15 medium	Eurobio	CM1L15000U	Culture medium for freezing solution
Mars-IVF Class II Workstation/L126 IVF Dual	CooperSurgical	WM1500/6-133-911-121	Workstation
Programmable freezer	Planner KRYO 500	Kryo 560-16	Other equipements may also be suitable

Scissors with sharp straight blades and finely sharpened points	Medlane	CI 034 03	Other material and sizes may also be suitable
Stripper	CooperSurgical	MXL3-STR-CGR	Other products may also be suitable
Tips (150µm) for Stripper	CooperSurgical	MXL3-150	Other products may also be suitable
Vitrification Kit	Irvine Scientific	90133	Protocols are available at http://www.irvinesci.com/products/90133-so-vitrification-freeze-solutions. Other products may also be suitable

Answers to reviewers

First, we would like to thank the Editor and all the Reviewers for their constructive comments and suggestions they made to improve the quality of our manuscript. We have made all the revisions as suggested and have answered all the questions. We believe this has significantly improved our paper.

Please note that all of the corrections in the manuscript will appear with green highlights.

In this document, our answers to editor's and reviewer's comments are in blue.

Editorial Comments

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

It has been done.

• Avoid punctuating the title and make it crisp.

The title has been modified.

• Please list a minimum of 6 keywords/phrases.

This has been done.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
The protocol has been completed.

Some examples:

1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.

A sentence has been added.

2) Section 1: mention inclusion and exclusion criteria.

A short paragraph has been added.

3) 5.2: how is the cutting performed?

A sentence has been added.

4) Line 248: Mention pipette tip size.

The sentence has been modified.

5) 9.8: How is vitrification done?

A sentence has been added and the table "JoVE Materials" (summarizing all the products used) has been modified. Detailed oocyte vitrification protocols for Irvine Scientific vitrification kit are available on line (http://www.irvinesci.com/products/90133-so-vitrification-freeze-solutions).

Hence, we have chosen not to detail the vitrification protocol in our manuscript. As other vitrification kits can also be chosen to perform the cryopreservation of oocytes according to the experience and constraints of each ART center, we think that describing the vitrification protocol in our manuscript is of no particular interest to the reader.

• **Protocol Numbering:**

1) Please add a one-line space after each protocol step.

This modification has been done.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

It has been done.

• Please upload each table as an individual Excel file.

It has been done.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion part has been modified.

• **References:**

1) Please spell out journal names.

This has been done.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are endobag,

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

The corrections have been made.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-

publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Our figures and tables are original.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The female perspective on fertility after cancer treatment is nowadays an important issue, in both young and adult patients. Providing a standardized fertility preservation protocol to increase the potential of fertility in prepubertal girls and women at risk of POI in the malignancy context is critical. Authors remind us that the available data on the developmental potential of oocytes derived from ovarian tissue are scarce, thus, further research is necessary.
The article provides a comprehensive description of the technique they applied in the study, that was optimized as recommended protocol. The described results are encouraging for adult females and have some drawbacks for the prepubertal girls. The preserved ovarian tissue is fit to be later used as oocites reserve or for the endocrine function. The discussion section offers sufficient information for specialists in the field.

Major Concerns:
None

Minor Concerns:
Authors declare that unilateral oophorectomy is recommendable, to avoid the use of any dissection device that could induce collateral electric or thermal injury to the ovarian tissue; still, surgical suturing is usable and efficient.

We thank the Reviewer for these comments. We have added a sentence about the dissection of ovarian tissue in the manuscript.

Reviewer #2:
Manuscript Summary:
The protocol is an alternative option for female fertility preservation at risk of premature ovarian insufficiency. It combines ovarian tissue freezing and cryopreservation of oocytes retrieved from ovarian tissue. This strategy might be good to women to improves the safety and optimizes the reproductive potential of fertility preservation, maximizing the chance of childbirth.

Major Concerns:
At present, the cryopreservation of mature oocytes is better than that of immature oocytes in terms of ART clinical outcomes. Why not perform IVM in this protocol?

This is a pertinent question; we thank the reviewer for this comment.

At present, the data are scarce in literature, preventing any conclusion on the most efficient strategy. Indeed, only one meta-analysis on this topic has been published in 2018 and it only concerns oocyte

maturation rate. This meta-analysis of 14 studies (Mohsenzadeh et al., 2018) reported that oocyte vitrification at GV stage had a significant negative impact on oocyte maturation rate (RR = 0.76, 95% CI: 0.66-0.88); I2 = 85.2%; P = 0.000)[1].

This meta-analysis has major limitations preventing any extrapolation for our protocol:

1) This meta-analysis has been published twice (Eur. J. Obstet. Gynecol. Reprod. Biol. in 2018 and Cryobiology in 2018), which casts doubt on the professionalism and the integrity of the team. A retraction notice has been published in 2019 in Eur. J. Obstet. Gynecol. Reprod. Biol. at the request of the Editorial Board as the paper contained a significant number of similarities and word overlap with the other paper published in Journal Cryobiology, representing duplication publication (see the retraction notice in: Mohsenzadeh M, Salehi-Abargouei A, Tabibnejad N, Karimi-Zarchi M, Ali Khalili M. Retraction notice to "Impact of vitrification on human oocytes before and after in vitro maturation: A systematic review and meta-analysis" [Eur. J. Obstet. Gynecol. Reprod. Biol. 227 (August) (2018), 19-26] [retraction of: Eur J Obstet Gynecol Reprod Biol. 2018 Aug;227:19-26]. Eur J Obstet Gynecol Reprod Biol. 2019).
2) Almost all of the studies included in this meta-analysis involved immature oocytes recovered after hormonal stimulation. These are therefore immature oocytes which have not responded to supra-physiological doses of FSH and hCG. One could speculate that they are poor quality oocytes, acting differently than immature oocytes that have never undergone supra-physiological hormonal stimulation.
3) These results derived from studies which used different vitrification medium (10/14 studies used in-house vitrification media). The authors noted that optimization of vitrification media may improve oocyte maturation rate after vitrification (Mohsenzadeh et al., 2018).
4) The included studies reported heterogenous results. Indeed, some of them (2/14 studies) showed that oocyte vitrification at GV stage significantly improves maturation rate by (RR = 1.32, 95% CI: 1.01–1.72) and (RR = 1.08, 95% CI: 0.99–1.19) respectively (Molina et al., 2016 and Zhang et al., 2017). And 4/14 studies showed no significant difference. Only one study used immature oocytes retrieved from ovarian tissue but it was obtained from patients undergoing gyneacological surgery for chocolate ovarian cysts. In this study, no significant differences were noted in the rates of maturation, fertilization and embryo development according to the timing of vitrification.
5) ART clinical outcomes (such as fertilization rate, embryo development rate, implantation/pregnancy and live birth rates) have not been analyzed in this meta-analysis. Hence, we cannot conclude that vitrification is better after IVM because the impact of vitrification on the IVM oocytes has not really been evaluated. Furthermore, only half of the included studies evaluated survival, cleavage, fertilization and blastocyst formation rates besides maturation rate. For example, Kasapi et al. reported that vitrification of in vitro matured MII oocytes was associated with increased rate of maturation but similar survival rate and similar incidence of normal spindle/chromosome configurations compared to GV oocytes matured after vitrification in stimulated cycles (Kasapi et al., 2017). As one major problem associated with cryopreservation of metaphase II oocytes is the sensitivity of the microtubular spindle to cryoprotectants and low temperatures[2], vitrification of immature oocytes at the GV stage could circumvent this problem.
6) Addition limitations of this meta-analysis are the small number of included studies along with the high heterogeneity between the involved studies due to the variation in the study design and lab techniques.
7) In addition to all these limitations, our strongest concern is the fact that the same IVM protocol was used in these studies before and after the vitrification/thawing step. Indeed, our strategy is based on the fact that frozen oocytes for fertility preservation will not be used for several years or even decades in the case of prepubertal girls. During this time, progress will most certainly be made in IVM protocols, giving hope for better results in terms of oocyte maturation rate and developmental/implantation potential.

8) Thus, for all of these reasons, we think that no study is available in the literature to conclude with certainty on the most efficient vitrification strategy (before or after IVM) to maintain the development and implantation potential of immature oocytes in the context of ovarian tissue cryopreservation. Hence, we currently prefer to freeze immature oocytes and not in vitro matured oocytes with a standard IVM protocol. We are convinced that some progress (even minor) will be made in the coming years, whether in IVM protocols or even in culture conditions (oocytes matured *in vitro* undergo 24-48 hours of culture contrary to immature oocytes immediately frozen after the retrieval from ovarian tissue). Obviously, our position could change in future depending on the evolution of the literature.

Minor Concerns:

1. Please describe the protocol of sterilization process of medical devices for ovarian tissue cortical treatment and the types of medical devices in the section of Quality control.

The protocol of sterilization process of medical devices is:

- The medical devices are washed in a disinfector washer combining a mechanical and a chemical cleanings and then one stage of thermal disinfection at 90°C for 5 minutes.
- Then, the medical devices undergo functional, cleanliness and dryness controls.
- The medical devices are then packaged in plastic trays with a specific sterilization package.
- It is then placed in the autoclave where it will be sterilized by water vapour at 134°C, for 18 minutes.
- If this step has gone smoothly, the tray will be labeled with an expiration date of 3 months.
- After cooling, the tray will be over-packaged in a plastic bag and transported to the laboratory.

We chose not to detail the sterilization protocol in our manuscript as it may vary depending on the institution. In addition, some laboratories may prefer to use single-use equipment. We have added a sentence on this issue in the manuscript in the section of Quality control.

Additional data on the types of medical devices are available in the manuscript and the Table « Jove Materials ».

2. It is better to present the average number of oocytes retrieved or crepreserved per prepubertal girls and per woman in the table 1 and table 2.

The correction has been made.

3. It is better to present the average number of oocytes retrieved per whole ovary or partial ovarian tissues in table 1.

The correction has been made.

4. Is there heparin sodium or antibiotic in IVF culture medium? How to pre-equilibrate IVF medium? What is the pre-equilibrating time?

We don't add any heparin sodium or antibiotic in any culture medium in our protocol. On the product leaflet, we can see that the IVF medium that we use (Continuous Single Culture Complete with HAS, Irvine Scientific) already contains Gentamicin sulfate. Additional information has been added on the pre-equilibrating step of IVF medium (see section 2).

5. What is the culture medium (Lines 177, 178,183)?

   This is Leibovitz L15 medium. The readers can find this information in the Table "JoVE Materials":

Leibovitz L15 medium	Eurobio	CM1L15000U	Culture medium for ovarian tissue collection, transport and tissue dissection

Reviewer #3:

Manuscript Summary:

Establishment of fertility preservation in prepubertal girls and women at risk of POI is important. The authors attempted to show their combined method of OTC and oocyte vitrification as an optimized method. Whilst interesting, there are several concerns to lead their conclusions.

Major Concerns:

1. In this manuscript, no clinical outcome was shown using frozen-thawed ovarian tissues and oocytes. How did the authors optimize the protocol without the data of clinical outcome?

We agree with the reviewer. Unfortunately, no clinical outcome using frozen-thawed ovarian tissues or oocytes has been evaluated to date in our study. This is the principle of fertility preservation, the use is shifted over time from a few years to a few decades, which complicates the analysis of the results.

However:

1) We have investigated the survival of immature oocytes enclosed in fresh and in frozen/thawed primordial, primary, and secondary follicles retrieved from the dissection spent medium as well as from the tissue in patients with no history of gonadotoxic treatment (see figure below). We noted a high rate of oocyte viability in fresh primordial/primary/secondary follicles (>90%) as well as in frozen/thawed primordial follicles (>80%). We have compared these results with the viability of immature follicles contained within the fixed tissue sent to the anatomopathologist at the end of our protocol (see section 8 of the manuscript) and conclude that our protocol did not cause follicular death. We have chosen not to include these controls in the publication because it does not demonstrate with certainty the absence of deleterious impact on immature oocytes obtained from antral follicles. Nevertheless, these results are reassuring.

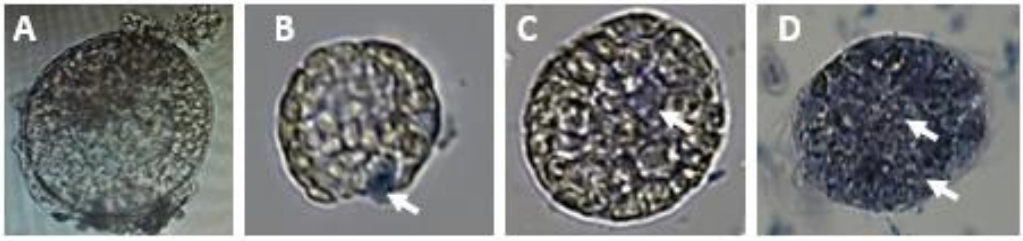

Figure 1: viability assessment of immature follicles retrieved from the dissection medium. Follicles with bright and homogeneous follicular cells around an intact oocyte were classified as morphologically normal[3]. Trypan blue staining was used to evaluate the viability of the follicles[4,5]. Dead cells are stained blue (white arrows), and live ones are unstained. A follicle was classified as dead if the oocyte was dead or denuded >25% of the surface, or >50% of follicular cells were dead. A-B) Viable follicles. C) Dead oocyte. D) Dead follicle.

2) Moreover, the rate of atretic oocytes retrieved from antral follicles in patients with no history of gonadotoxic treatment is in coherence with the literature (between 10-40% in prepubertal girls[6] and around 12% in immature oocytes retrieved in women in non-primed IVM cycles[7]), suggesting again that our protocol did not cause follicular death.
3) The numbers of oocytes retrieved from the tissue during OTC are associated with the mean follicle densities measured by the histological analysis of the ovarian tissue samples sent to the Anatomopathologist (i.e. Step 8) and we found no antral follicle in the medulla or cortical tissues after the dissection step, suggesting that the efficiency of our protocol is satisfactory and that the extreme majority of immature oocytes from antral follicles are retrieved with our dissection protocol.
4) We plan to conduct a study on oocytes and ovarian tissues frozen with our protocol and then donated to the research. At present, only one patient has donated her tissue and oocytes to the research (1/38 patients, Supplementary Table 1), which allow us to consider future experiments to measure the effectiveness of our protocol. We are awaiting further research donations of patients who have benefited from cryopreservation of ovarian tissue and immature oocytes (e.g. from patients who have succeeded in conceiving without the use of their cryopreserved tissue or oocytes; or from patients who finally do not want children) to begin a research protocol evaluating the quality of the cryopreserved oocytes and ovarian tissue. While waiting for this

study to be carried out, we have removed the notion of "optimized protocol" from the manuscript.

2. Although the authors discussed why they cryopreserved immature oocytes without IVM, it is unclear which is better cryopreservation of oocytes before or after IVM based on their own data.

We don't have own data on this subject. We have based our position on the current literature and the possibility of progress in IVF protocols in the coming years (please see our response to Reviewer #2 for more information). Currently, there is a lack of published data based on retrieved oocytes from ovarian tissue to answer to this question. To our knowledge, the only study available has been published in 2001 and performed on oocytes retrieved from ovarian tissue containing chocolate ovarian cysts[8]. The authors reported no significant differences in the rates of maturation, fertilization and embryo development according to the timing of vitrification and IVM. Of course, well-conducted studies are still needed to conclude[8]. Anyway, our choice is based on the fact that frozen oocytes for fertility preservation will not be used for several years for woman or even decades in the case of prepubertal girls. During this time, we think that progress will most certainly be made in IVM protocols or culture conditions (e.g. incubators, 3D culture system for IVM etc.), giving hope for better results in terms of oocyte maturation rate and developmental/implantation potential (please see our response to Reviewer #2 for more information).

3. Current introduction is redundant and too long. It needs to fit the objectives of JoVE journal.

The corrections have been made.

4. The authors performed oocyte denudation at two hours after COCs isolation. How did they decide this incubation time?

We thank the reviewer for this constructive comment. To our knowledge, there is no data on the optimal time between the isolation of COCs from the ovarian tissue and the denudation of the oocytes in the context of fertility preservation. Nevertheless, there are some data in the literature on the optimal time between the oocyte pick-up (OPU) and the denudation of the oocytes in the context of ovarian stimulation. The first publications suggested that oocyte incubation periods of 2–3 h from OPU to ICSI could improve fertilization rates and quality of the derived embryos[9-12]. Later, another publication corroborates these first results by reporting that incubation of oocytes around 2 hours between OPU and denudation may not increase MII rate but appears to lead to the optimal combination of fertilization rate and implantation rate[13]. All of these publications explain our initial choice to carry out an incubation of 2 hours between the OPU and the denudation in our protocol. However, recent literature does not support these initial results. Indeed, several reports observed no significant differences in reproductive outcomes of ICSI cycles within a wide range of times between OPU and oocyte denudation[14-16]. Moreover, Pujol et al. recently suggested that increasing OPU-ICSI time could increase the fertilization rate but decrease the likelihood of biochemical pregnancy after the fresh ET[17]. As a consequence, we remove this part in the manuscript (Part 9.1).

Minor Concerns:

1. Abstract and discussion: no data about in vitro follicle growth in three-dimensional systems was demonstrated in this manuscript. Thus, corresponding parts of text should be deleted.

We have deleted these parts.

2. In this method, the upper limit of time for transport ovarian tissue to the IVF laboratory is unclear.

We thank the reviewer for this constructive comment. We recommend to transport the ovarian tissue to the laboratory as soon as the sample has been taken (as soon as the tissue has been removed from the ovary). If necessary, the ovarian tissue can be transport at cold temperatures (around 4°C) to the laboratory for up to 26 hours after oophorectomy without threatening the quality of it as assessed by morphology, viability, and follicle development *in vitro* and *in vivo*[18]. This is particularly reassuring in the

case of ovarian cryopreservation performed on two geographically distant sites (different centers, different cities, see different countries). This information has been added to the manuscript.

3. What kind of IVF culture medium did the authors use?

This is Continuous Single Culture Complete with HAS, Irvine Scientific. The readers can now find this information in the Table "JoVE Materials":

Continuous Single Culture Complete with HSA	Irvine Scientific	90165	IVF culture medium for follicular fluid collection, COCs incubation, oocyte denudation and oocyte incubation until the vitrification step.

5. The definition of healthy COC is unclear.

We thank the Reviewer for this comment. Indeed, this notion required some clarification. Representative pictures of healthy (A-B1-B2-B3) and unhealthy (C1-C2) COCs are presented in Figure 2. A definition of healthy COCs has been added in the manuscript (Part 5.5). The notion of "healthy COC" is based on the color of the oocyte: translucent = healthy whereas brown = unhealthy. This definition is not too discriminating and this makes it possible to exclude only the COCs containing an atretic oocyte, which leaves the other COCs "a chance" to go to the denudation step to visualize the oocyte more precisely. Thus, the risk of error is minimized and no viable oocyte is mistakenly discarded.

6. Typo: line 165: 5-ports should be 5-mm ports.

The correction has been made.

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	Prepubertal girls n=43	Women n=38	Statistical analyses	P value
Storage	90.70% (39/43)	81.58% (31/38)	Fisher's Exact Test	0.33
Destruction	9.30% (4/43)	18.42% (7/38)		
Destruction following patient death	9.30% (4/43)	15.79% (6/38)	Fisher's Exact Test	1
Destruction following patient decision	0% (0/43)	2.6% (1/38)		
Loss to follow-up	4.65% (2/43)	10.53% (4/38)	Fisher's Exact Test	0.41