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Fertility preservation in the pediatric and adolescent populations, volume II?

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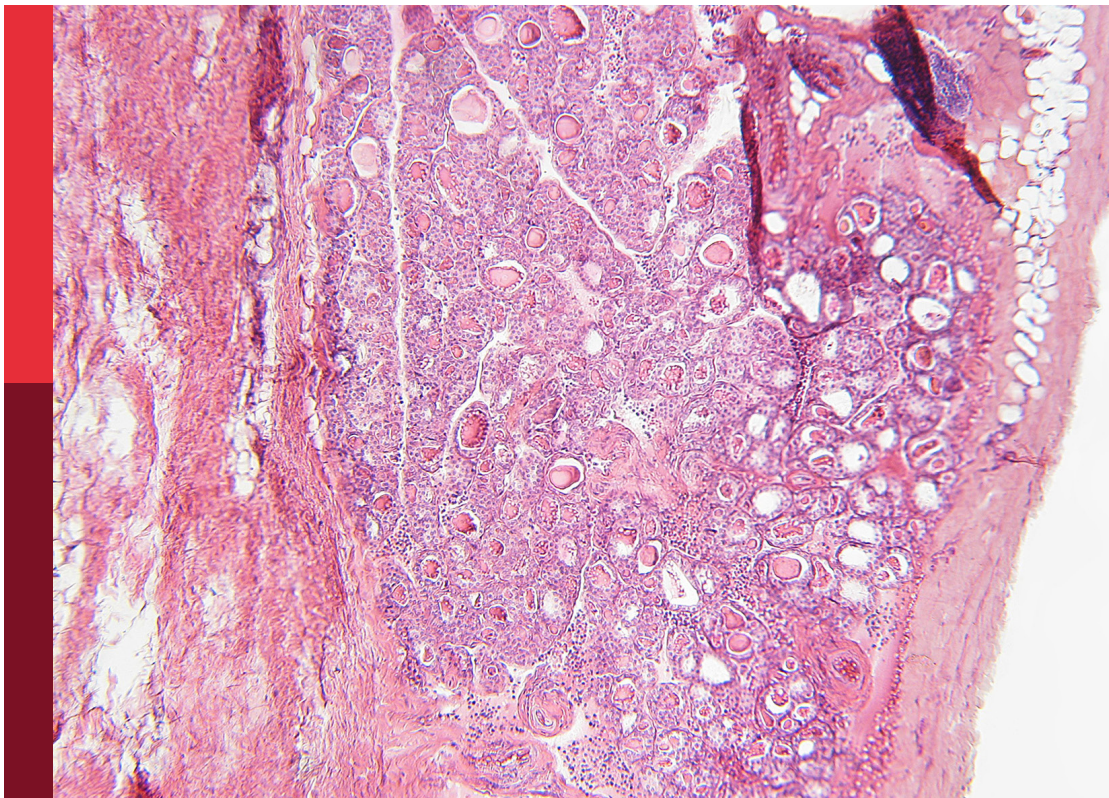

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Fertility preservation in the pediatric and adolescent populations, volume II?

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Editorial: Fertility preservation in the pediatric and adolescent populations, volume II

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Editorial on the Research Topic
Fertility preservation in the pediatric and adolescent populations, volume II

The Frontiers in Endocrinology Research Topic on fertility preservation (FP) in the pediatric and adolescent populations invited authors from across the globe to participate in the dissemination of knowledge and awareness regarding the best fertility preservation principles in the pediatric and adolescent populations.

This Research Topic serves as a dedicated research publication, or part thereof, to highlight the important aspects of missing research information in pediatric and adolescent fertility preservation programs and aims to advance the science considerably. Since many centers, nationally and internationally, are not even aware of best practice guidelines for pediatric and adolescent fertility preservation in children and adolescents facing fertility threatening diagnoses and treatment plans, disseminating knowledge regarding the same in both providers and thus the population at large, is much needed.

The importance of FP is being increasingly recognized, with many international guidelines now removing the lower age limit for offering FP (1–4). This Research Topic demonstrates the significant knowledge gains in oncofertility that continue to be achieved in the young population.

A review by Chen et al. demonstrates the continuing progress in fertility preservation technologies. Ovarian tissue cryopreservation is deemed innovative, however continues to advance with over 200 births estimated to have been achieved by 2020 (5), with average live birth rates around 23% (6). Consecutive pregnancies (the highest being four) from the same graft have been seen, making it a very efficient fertility preservation method (7). Research efforts to protect against malignant contamination after tissue grafting are ongoing. Recurrence of malignancy has been reported in 3.9-7%, and none thought to be related to the ovarian tissue grafting process (8, 9).

Recent cases of successful prepubertal oocyte collection have been reported (in a 7-year-old patient with Turner's mosaicism who collected 6 oocytes (10), and a transgender

male under 12 years who collected 9 oocytes) (11) raising questions about best fertility preservation options for young birth-assigned female patients. This is addressed further in the first systematic review of oocyte collection in 468 females and transgender males ≤ 18 years (median age 15.2 years) by Slonim et al.. The majority of stimulation cycles (96.3%) successfully obtained oocytes and complications were rare. The highest success was seen in the transgender population (compared to those about to receive gonadotoxic therapy or those with Turner syndrome). Only one live birth from cryopreserved oocytes has been reported in this age group (12), with the authors recommending that oocyte collection in post-pubertal adolescents be regarded as innovative, while for prepubertal patients it should be considered experimental due to unknown oocyte quality.

For birth-assigned males, over 1000 testicular tissue biopsies were reported by 2020 (13). However successful human birth after testicular tissue cryopreservation has not been seen. Following on from the success of the first primate birth in 2019 (14), Younis et al. describe *in vitro* maturation of immature testicular tissue from both pre and peripubertal males to primary spermatocyte stage after being maintained in organotypic culture for 32 days. While complete spermatogenesis was not seen, these findings provide increasing hope that by the time a prepubertal male reaches adulthood, realistic attempts at parenthood may be made.

Populations who can potentially benefit from fertility preservation procedures continue to expand. In this Research Topic, Rodriguez-Wallberg et al. reported on outcomes of 100 women with Turner syndrome and recommend referral for fertility preservation counselling after onset of puberty to maximize yield. Follicles were seen in 25% of ovarian tissue biopsies analyzed and were more likely present in adolescents compared to prepubertal children or adults. Similarly, reports on quality of ovarian and testicular biopsies in those with rare diagnoses such as mucopolysaccharidoses and Diamond Blackfan syndrome and disorders of sexual differentiation (frameshift mutations) are reported by Ruan et al. and Teoli et al. respectively, which can further help clinicians with fertility preservation counselling for these patients.

One of the most complex aspects of fertility preservation in children is the triadic nature of decision making (between the parent, patient and the clinician). The clinician plays an important role assessing comorbidities, mitigating risk and communicating clear and unbiased medical recommendations to families in order to support informed decision-making. Parents who are the surrogate decision makers for younger children may experience conflict and concern around making choices that might be incongruent with the child's future wishes (15). In this new Frontiers Research Topic Takae et al. report findings from a novel study examining comprehension and attitudes of children and adolescents towards fertility preservation before and after age-appropriate explanation, tailored to the understanding of the individual child. This included the use of storytelling for very

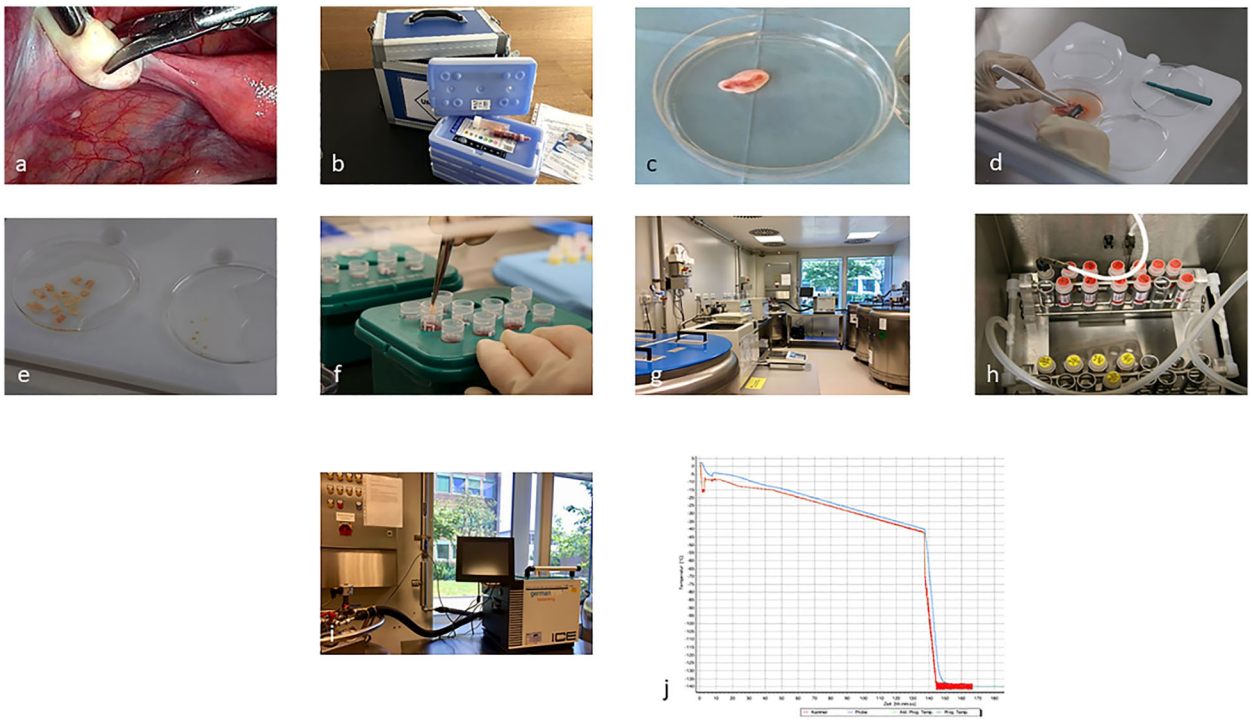

FIGURE 1
Ovarian tissue cryopreservation process from laparoscopic biopsy **(A)**, (overnight) transport **(B)**, to preparation and freezing of the cortical biopsies **(C–J)** (Baston-Büst et al.).

young children. The majority of participants were females with median age of 14 (6-17) years. Before the intervention, the majority of children under 11 years (63.4%) did not know if they wanted children in the future and were non-committal about learning about fertility preservation options, but this changed after education, with over 90% wanting to hear more about fertility preservation options. While children should not be relied upon to take responsibility or consent to fertility preservation procedures, for those who are submature and have some understanding, it is appropriate to have their agreement or at least not have their disagreement prior to having a surgical intervention.

Finally, implementation of fertility preservation programs and cryobanks is another priority in order to address disparities in care (16). In this Research Topic Baston-Büst et al. discuss their step-wise approach for implementing a cryobank in Germany in a university-based setting, including engagement of key-stakeholders and development of standard operating procedures for ovarian tissue cryopreservation (Figure 1). Quality assurance and audit is a regulation in most laboratories but should also be part of any clinical fertility preservation program through the development of national oncofertility registries to enable collaboration and meaningful evaluation of long-term outcomes of fertility preservation interventions. Accordingly, Ozimek et al. concluded that although oncofertility services are expanding globally, very few countries have well-established official national oncofertility registries. The authors highlight the urgent need for having a well-established official national oncofertility registry in each country to monitor oncofertility services in a way that best serves patients.

Author contributions

MS: Writing – original draft, Writing – review & editing. YJ: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. YJ is funded by the Royal Children's Hospital Foundation and the Medical Research Future fund MRFAR0000308.

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Survey of understanding and awareness of fertility preservation in pediatric patients: Is conversation about fertility preservation unpleasant for pediatric patients?

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Objective: To verify understanding and awareness of fertility preservation (FP) in pediatric patients undergoing FP treatments.

Methods: A questionnaire survey was conducted before and after explanation of fertility issues and FP treatments for patients 6–17 years old who visited or were hospitalized for the purpose of ovarian tissue cryopreservation (OTC) or oocyte cryopreservation (OC), or sperm cryopreservation between October 2018 and April 2022. This study was approved by the institutional review board at St. Marianna University School of Medicine (No. 4123, UMIN000046125).

Result: Participants in the study comprised 36 children (34 girls, 2 boys). Overall mean age was 13.3 ± 3.0 years. The underlying diseases were diverse, with leukemia in 14 patients (38.9%), brain tumor in 4 patients (11.1%). The questionnaire survey before the explanation showed that 19 patients (52.8%) wanted to have children in the future, but 15 (41.7%) were unsure of future wishes to raise children. And most children expressed some degree of

understanding of the treatment being planned for the underlying disease (34, 94.4%). Similarly, most children understood that the treatment would affect their fertility (33, 91.7%). When asked if they would like to hear a story about how to become a mother or father after FP which including information of FP, half answered "Don't mind" (18, 50.0%). After being provided with information about FP treatment, all participants answered that they understood the adverse effects on fertility of treatments for the underlying disease. Regarding FP treatment, 32 children (88.9%) expressed understanding for FP and 26 (72.2%) wished to receive FP. "Fear" and "Pain" and "Costs" were frequently cited as concerns about FP. Following explanations, 33 children (91.7%) answered "Happy I heard the story" and no children answered, "Wish I hadn't heard the story". Finally, 28 of the 34 girls (82.4%) underwent OTC and one girl underwent OC.

Discussion: The fact that all patients responded positively to the explanations of FP treatment is very informative. This is considered largely attributable to the patients themselves being involved in the decision-making process for FP.

Conclusions: Explanations of FP for children appear valid if age-appropriate explanations are provided.

KEYWORDS

fertility preservation, pediatric cancer, ovarian tissue cryopreservation, oocyte cryopreservation, oncofertility, children's response to fertility preservation explanations

1 Introduction

In recent years, interest in post-treatment quality of life and the late complications of cancer survivors has been increasing. Furthermore, with the aim of popularizing and developing fertility preservation (FP), the "Oncofertility Consortium" and "FertiPROTEKT" were established in 2006, followed by the International Society for Fertility Preservation in 2009, the Japan Society for Fertility Preservation (JSFP) in 2012, and the Asian Society for Fertility Preservation in 2015 (1). The importance of FP has also been recognized more widely in society, and public financial support for FP became available in Japan in 2021. The number of patients desiring FP is thus expected to increase in the future. In particular, the number of cases involving prepubertal children, for whom FP has been difficult to implement, seems likely to increase markedly. Further, major organizations such as the American Society of Clinical Oncology (2) (3) (4),,, European Society of Medical Oncology (5), and European Society of Human Reproduction and Embryology (6) have all published FP guidelines, and in Japan, the Japan Society of Clinical Oncology and JSFP have jointly published FP guidelines (7) and were revising these guidelines as of October 2022. These advanced guidelines have eliminated lower age limits, but do not provide specific methods for implementing FP in infants and prepubertal children (8). This is understandable, since FP initially continued to develop technologically for adults such as breast cancer patients. However, since pediatric patients with blood diseases are increasingly major targets of FP measures, clarifying specific methods of dealing with pediatric patients is crucial. In fact, the spread of FP to pediatric patients is reportedly delayed in Asia compared to adult patients, due to difficulties with providing information, a lack of explanatory materials, and lack of cooperation with pediatricians (1). In addition, when a child receives information about FP in the first place, the intention of the parent has a substantial influence, and the parent may act as a barrier and not provide information to the child. Unsurprisingly, parents consider various factors such as the age of the child, the degree of intellectual development, context and timeline for decision-making, costs, the mental health of the child, the invasiveness of potential FP interventions, and culture. At some point, medical staff may finally arrive at the point of providing information on FP to the child (9). Few clinical studies

Abbreviations: FP, fertility preservation; OC, oocyte cryopreservation; OTC, ovarian tissue cryopreservation; GnRHa, gonadotropin releasing hormone agonist.

with comparisons to adults have been conducted, and no reports have described the psychological problems and comprehension of pediatric patients themselves due to the difficulties inherent in conducting such investigations.

We therefore undertook a preliminary survey on the perception of FP in pediatric patients, the degree of understanding of FP after provision of an explanation, and feelings toward FP. We believe that this survey will provide basic insights into the actual feelings of pediatric patients and should contribute to the implementation of high-quality FP for pediatric patients.

2 Materials and methods

2.1 Patients

Participants in this study were children between 6 and 17 years old who visited our hospital for the purposes of FP consultation or who were hospitalized for the purpose of OTC between October 2018 and July 2022. All children had a malignant disease such as leukemia or a disease such as aplastic anemia or chronic active Epstein-Barr virus (EBV) infection, in which there was a possibility that treatment of the primary disease with chemotherapy and/or radiotherapy would greatly impair future fertility.

2.2 Explanation of FP

Before providing an explanation of FP, we gave the parents and child a leaflet created by the JSFP. After reading the leaflet, the parents and child were given an explanation about FP alike telling a story (suppl. 1). However, we did not verify whether parents had told the child about the contents of the leaflet before the explanation. During the actual explanation, a male FP doctor specializing in reproductive medicine (including FP) and endoscopic surgery subjectively evaluated the development status of child while talking with the child and explaining FP by drawing pictures on a piece of paper. So, the explanation of the FP seemed to tell the story. Moreover, it was remarkable when explaining to younger children. Also, he and young female assistant doctors also provided explanation using an original animated movie currently under development as a supplement. The method of explanation was changed taking into consideration not only age but also comprehension. Basically, after having girls in their teens and beyond puberty read the leaflet, we explained it including a certain degree of specialized knowledge. For elementary school students, we asked their parents to explain without reading the leaflet, and for younger children, we mainly explained with parable. When the subject of the explanation for FP was a girl, consideration was given to female doctors and nurses in attendance as much as possible, so that the explanation would not be given only by male doctors.

2.3 Questionnaire survey

Before and after the explanation of FP, a questionnaire survey was conducted to evaluate the feelings and perceptions of the child. There was no age-specific version of the survey, and for children who could not read or write sufficiently, parents explained the content and filled it out. Children who can read and write on their own were basically included while confirming the content with their parents. Most of the children after puberty checked the contents by themselves and answered by themselves. Therefore, it cannot be denied that the reliability of this survey declines with low age. The contents of questions before the explanation of FP consisted of 10 questions, including the sex and age of the child. The contents were "Do you know about the planned treatment for your illness?", "Do you know that treating your illness may make it harder for you to become a mother or father in the future?", and so on. Details of the questions before providing the explanation about FP are shown in Table 1. The number of questions posed after the explanation about FP was smaller than before the explanation, in consideration of the physical and mental burden of the child. There were 8 questions, with the contents designed to elucidate changes in knowledge, such as "Do you understand the effect of treatment for your illness on your chances of being a mother or father in the future?", and "Do you understand the medical technology to preserve your chances of being a mother or father in the future?", and so on. In addition, questions such as "Do you want to receive medical technology to preserve your chances of being a mother or father in the future?", and "After these explanations, have you noticed any change in your desire to become a mother or father?", were posed. Questions asking about the psychological state of the child, such as "Please tell us how you felt after listening to the story." were also set. Details of the questions after the explanation about FP are shown in Table 2. The questionnaires used in this study were evaluated and modified by the researchers and psychologists specializing in reproductive medicine and FP.

2.4 Ethical considerations

Since the content of the questions set in this study was very sensitive, the questionnaire was filled out with the parents after guaranteeing the right to refuse to answer at any time. In addition, the questionnaire was conducted under careful observation by the medical staff to see if the child exhibited

TABLE 1 Questionnaire pre-explanation of fertility preservation.

Pre	Questions	Answers
Q1	Please tell me your gender.	(boy, girl)
Q2	How old are you?	() years old
Q3	Do you want to have your own children when you grow up? (Would you like to be a mother or father)?	(I really think so, I think so, I don't know yet, I don't think so, I really don't think so)
Q4	Do you know about the planned treatment for your illness?	(Know well, Know, Know a little, Don't really know, Don't know anything)
Q5*	Please indicate what you know about treatment for Q4.	(Surgery, Chemotherapy (drugs), Radiotherapy, Hematopoietic cell transplantation, Other, Don't know)
Q6	Do you know that treating your illness may make it harder for you to become a mother or father in the future?	(Know well, Know, Know a little, Don't really know, Don't know anything)
Q7	Do you know what you came to hear?	(Know well, Know, Know a little, Don't really know, Don't know anything)
Q8	Would you like to hear a story about how to become a mother or father in the future (after FP)?	(Really want to hear, Want to hear, Don't mind, Don't really want to hear, Don't want to hear at all)
Q9	Do you know that there are medical technologies that can help you become a mother or father in the future?	(Know well, Know, Know a little, Don't really know, Don't know anything)
Q10*	Please indicate what treatments you know for Q9.	(OC, OTC, Ovarian suppression, Sperm cryopreservation, Testicular tissue cryopreservation, Gonadal shielding, Don't know)
		*Multiple selections allowed

Before providing an explanation about fertility preservation, knowledge about the treatment for the underlying disease of the child and knowledge about fertility preservation were examined.
OC, oocyte cryopreservation; OTC, ovarian tissue cryopreservation; FP, fertility preservation.

any physical or mental changes while answering the questionnaire. This study was conducted under the approval of the Institutional Review Board at St. Marianna University School of Medicine (approval no. 4123, UMIN000046125).

3 Results

3.1 Characteristics of patients

A total of 36 pediatric patients participated in this study, with a response rate of 100%. All children and parents agreed to participate in this study. Thirty-four of the 36 patients were girls and the other 2 were boys. The overall median age was 13.3 ± 3.0 years old. The median age of girls was 14 years (range, 6–17 years). The boys were 14 and 15 years old and the primary illness in both cases was ALL. Of these 36 patients, 11 were <11 years old (Group A), 9 were 12–14 years old (Group B), and 16 were 15–17 years old (Group C). Figure 1 shows the age distribution of study participants. In addition, the primary diseases of girls were diverse, with 14 leukemias (38.9%), 4 brain tumors (11.1%), 4 rhabdomyosarcomas (11.1%), 4 other sarcomas (11.1%, such as Ewing's sarcoma and osteosarcoma), and 3 malignant lymphomas (8.3%). In addition, anaplastic anemia was present in 2 children, and myelodysplastic syndrome (MDS), mediastinal tumors, systemic lupus erythematosus (SLE), chronic active Epstein-Barr virus infection (CAEBV), and thalassemia in 1 patient each. Most were primary cases, but two patients (including one boy) were relapse cases. In addition, 25 patients (69.4%) were already undergoing chemotherapy at the time of their visit, and most of the remaining patients had already undergone surgery, immunosuppressant therapy, blood transfusion therapy, etc.; only one patient was completely untreated.

As a result of the explanation about FP, two boys chose sperm cryopreservation, but one was already suffering chemotherapy-induced azoospermia. In addition, 28 girls (82.4%) underwent OTC and one underwent OC, whereas two girls thought that treatment of the underlying disease would have no significant effect on their own ovarian reserve and decided to follow-up on ovarian reserve. Notably, one girl initially chose OTC and was hospitalized, but her feelings changed immediately before the procedure and she eventually declined to undergo OTC. In addition, two girls prioritized

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TABLE 2 Questionnaire post-explanation of fertility preservation.

Post	Questions	Answers
Q1	Do you understand the effect of treatment for your illness on your chances of being a mother or father in the future?	(Understand well, Understand, Understand a little, Don't really understand, Don't understand at all)
Q2	Do you understand the medical technology to preserve your chances of being a mother or father in the future?	(Understand well, Understand, Understand a little, Don't really understand, Don't understand at all)
Q3	Do you want to receive medical technology to preserve your chances of being a mother or father in the future?	(Really want to receive FP treatment, Maybe want to receive FP treatment, Don't know, Don't really want to receive FP treatment, Don't want to receive FP treatment at all)
Q4	Please indicate the medical technologies you may receive.	(OC, OTC, Ovarian suppression with GnRH agonist, Sperm cryopreservation, Testicular tissue cryopreservation, Gonadal shielding against radiation, Don't really understand)
Q5*	What are your concerns about receiving the treatment you selected in Q4?	(Fear, Worried about pain, Not sure what to do, Worried about costs, Don't want to be transferred, Don't understand the need for FP, Don't want to receive any more burdensome treatments, Worried but unsure why)
Q6	After these explanations, have you noticed any change in your desire to become a mother or father?	(Yes, No, Don't know)
Q7	If there were any changes in Q6, please tell us what they are.	(Greater desire to have children, Greater desire to not have children, Other)
Q8	Please tell us how you felt after listening to the story.	(Happy I heard the story, Wish I hadn't heard the story, Didn't really understand, Other)
		*Multiple selections allowed

After receiving the explanation about fertility preservation, the reactions, concerns, and impressions of the child to the explanation were examined.
OC, oocyte cryopreservation; OTC, ovarian tissue cryopreservation; FP, fertility preservation, GnRH, gonadotropin-releasing hormone.

treatment of the underlying disease and did not choose any FP. Generally, at our institution, OTC is performed by single-port laparoscopic surgery, and the most important thing is to perform minimally invasive and safe procedures based on the policy of reduced-port surgery.

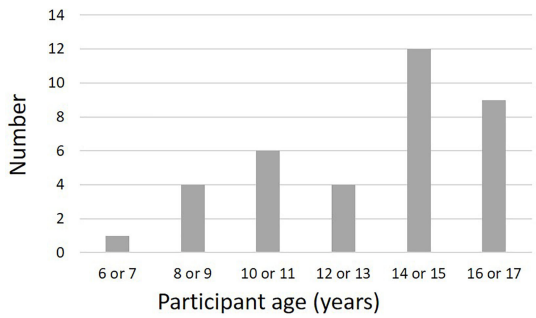

FIGURE 1
Age distribution of participants in the present study. The peak age groups were 14–15 years and 16–17 years. Participants also included 11 prepubertal children ≤11 years old. The youngest participant was 6 years old.

3.2 Questionnaire survey results before explanation about FP

All children were able to answer about their age and biological sex (Q1 and 2). Regarding Q3 (desire to have children in the future), more than half of Groups B and C answered, "I really think so" or "I think so". However, although no negative answers were seen in the younger Group A, 7 children (63.6%) answered that "I don't know yet" (Figure 2A). Regarding Q4, in Groups B and C, patients knew the treatment for the underlying disease that they were planning to receive, but in Group A, 5 of the 11 respondents (45.5%) said they "Know a little". A small number of children were unaware of the treatment being planned (one patient each in Groups A and C) (Figure 2B). Q5 asked for details about the subsequent planned treatment. Of the 36 patients, 17 (47.2%) responded that the treatment would be surgical, 21 (58.3%) responded chemotherapy, 12 (33.3%) responded radiation therapy, and 8 (22.2%) responded hematopoietic cell transplantation (multiple answers allowed). In addition, most children understood that treatment for the underlying disease would adversely affect fertility (Q6), with 8 of 11 (72.2%) in younger Group A, 8 of 9 (88.9%) in Group B, and 12 of 16 (75%) in Group C answering that they "Know well" or "Know". In Group C, which is the

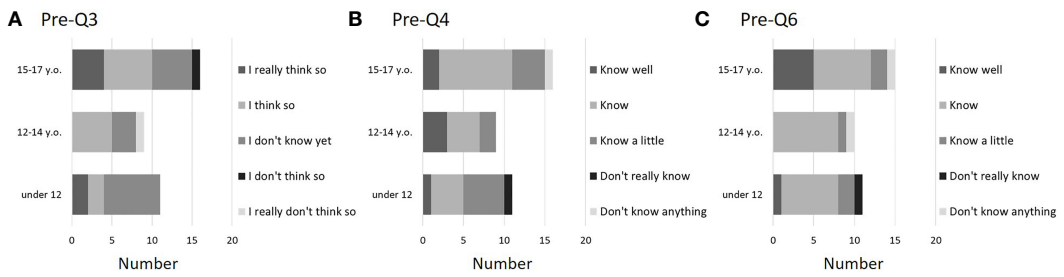

FIGURE 2
Questionnaire results before FP explanation (pre-Q3, 4, and 6). About half of the affected children expressed hopes of becoming parents in the future, but some (especially young children) were unsure of their feelings about "wanting to become father or mother" (**A**: pre-Q3). In addition, some children (particularly among young children) did not understand the planned treatment (**B**: pre-Q4). On the other hand, children tended to gain an understanding of the effects of disease treatment on fertility and the reasons for their visit (**C**: pre-Q6). Of these 36 patients, 11 were <11 years old (Group A), 9 were 12–14 years old (Group B), and 16 were 15–17 years old (Group C).

upper grades, the percentage of those answering "Know well" was high, at 5 of 16 children (31.2%). One child in each age group answered "Don't really know" or "Don't know anything" (Figure 2C). In Q7, which asked about the purpose of coming to our hospital, most children answered that they "Know well", "Know", or "Know a little" (Figure 3A). Regarding Q8, which asked whether they would like to hear explanation about the process from FP to assisted reproductive medicine, less than half of the children answered "Really want to hear" or "Want to hear", and many answered "Don't mind" (Figure 3B). This tendency was particularly noticeable in Groups A and B, who were younger, with 6 of 11 (54.5%) children in Group A and 5 of 9 (55.6%) children in Group B responding "Don't mind". Many children were aware of the existence of FP treatment (Q9), especially among the older children in Group C, and all were at least somewhat aware (Figure 3C). Regarding Q10, the most common FP treatments they knew of were OC (21 of 36, 58.3%) and OTC (18 of 36, 50.0%). Five patients were also aware of sperm cryopreservation (13.9%), including one boy, and three were aware of testicular tissue cryopreservation. All 4 girls who knew about sperm cryopreservation and all 3 girls who knew about testicular tissue cryopreservation were Group C girls. Regarding medical terminology, were given to the children by medical staffs or parents as appropriate. Therefore, there were no major difficulties in conducting present clinical studies

3.3 Questionnaire survey results after explanation about FP

After the explanation, most children understood the effects on fertility of the treatment for the underlying disease (post-Q1). None of the children answered, "Don't really understand" or "Don't understand at all" (Figure 4A). Regarding FP treatment, everyone answered that they understood to some degree, including "Understand a little" (post-Q2) (Figure 4B). When asked if they

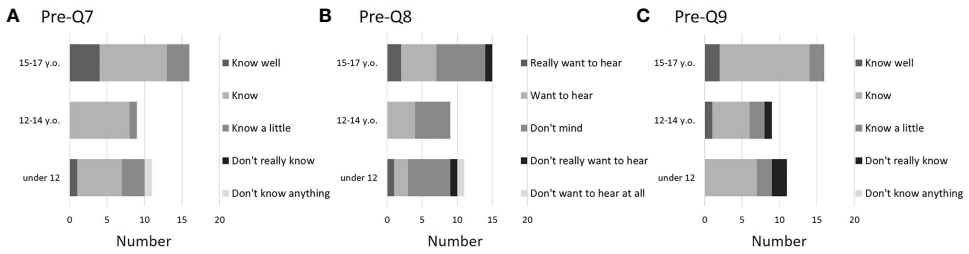

FIGURE 3
Questionnaire results before FP explanation (pre-Q7, 8, and 9). Similar to pre-Q6, many children recognized the reasons for visiting our hospital (**A**: pre-Q7). However, not many children were enthusiastic about hearing a story about becoming a parent in the future, consistent with Q3 (**B**: pre-Q8). In addition, many children were aware of FP treatment, which was generally consistent with pre-Q6 and -Q7(**C**: pre-Q9). Of these 36 patients, 11 were <12 years old (Group A), 9 were 12–14 years old (Group B), and 16 were 15–17 years old (Group C).

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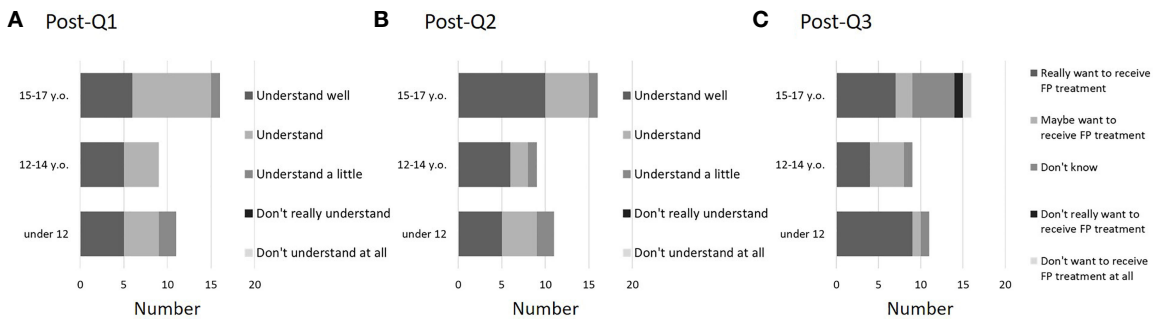

FIGURE 4
Questionnaire results after FP explanation (post-Q1, 2, and 3). A certain degree of understanding was reported, and no patients reported no understanding at all of the effects of treatment on fertility or of FP treatment (**A**: post-Q1; **B**: post-Q2). In addition, two participants aged 15–17 answered that they did not want to undergo FP, but one (a boy) subsequently decided to undergo sperm cryopreservation. The other (a girl) did not meet the indications for FP (**C**: post-Q3). Of these 36 patients, 11 were <11 years old (Group A), 9 were 12–14 years old (Group B), and 16 were 15–17 years old (Group C).

would like to receive FP treatment (post-Q3), 10 of 11 children (90.9%) in Group A, 8 of 9 children (88.9%) in Group B, and 9 of 16 (56.3%) in Group C answered they "Really want to receive FP treatment" or "Maybe want to receive FP treatment". In addition, a girl who answered that "Don't want to receive FP treatment at all" was not indicated for FP treatment because the treatment for the primary disease had not so large effect on fertility. A boy who answered "Don't really want to receive FP treatment" underwent sperm cryopreservation (Figure 4C). Regarding FP treatments that they may receive (post-Q4), 7 of 36 children (19.4%) responded "OC", 30 of 36 (83.3%) responded "OTC", 2 responded "Sperm cryopreservation", and 2 responded "Ovarian suppression with GnRH agonist". In addition, no children answered, "Testicular tissue cryopreservation" or "Gonadal shielding against radiation", but 3 of 36 (8.3%) responded that "Don't really understand" (2 in Group A and 1 in Group C). Post-Q5 asked about anxiety factors related to FP treatment. Ten of the 36 children (27.8%) did not answer this question (Figure 5A). The most common anxiety factor was "Fear" (14 of 36, 38.9%), followed by "Worried about pain" (13 of 36, 36.1%). The next most common answer was "Worried about costs". Two of 11 respondents (18.1%) in Group A and 5 of 16 (31.3%) in Group C answered "Worried about costs". After

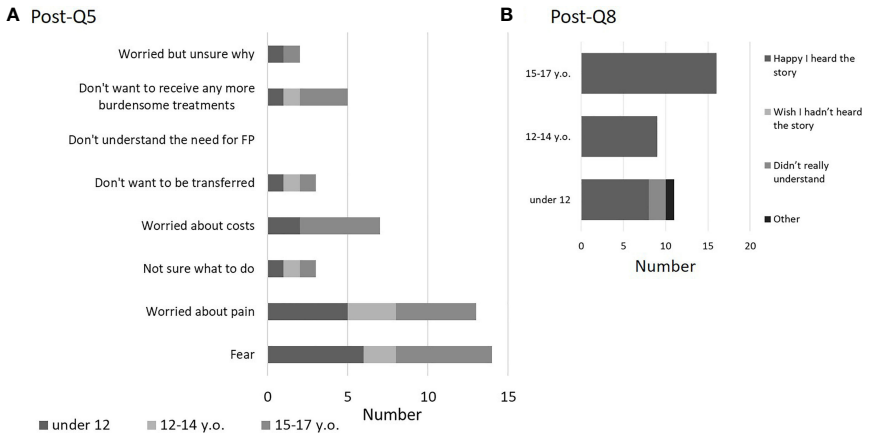

FIGURE 5
Questionnaire results after FP explanation (post-Q5 and 8). Key concerns about FP involved fear and pain, followed by financial concerns. No age-related differences were seen in these results (**A**: post-Q5). After the explanation, all children showed a positive reaction. The results also included patients (5 girls) who did not receive FP and the patients (one girl and boy) who responded that they did not wish to undergo FP (**B**: post- Q8). Of these 36 patients, 11 were <11 years old (Group A), 9 were 12–14 years old (Group B), and 16 were 15–17 years old (Group C).

explaining about FP, only 3 children reported a change in their desire to have children, with a positive change in 2 of the 3 children, and no response in the remaining (post-Q6 and 7). Finally, in post-Q8, which asked about impressions of the explanations for FP, 33 of the 36 children (91.7%) answered "Happy I heard the story" and 2 children (6- and 11-year-old girls) from Group A answered, "Didn't really understand". None of the children answered, "Wish I hadn't heard the story" (Figure 5B).

4 Discussion

FP treatment for pediatric patients has gained popularity in recent years, and the number of cases has been increasing (10–14). This trend is spreading not only in Europe and the United States, but also throughout Asia and worldwide (15, 16). In addition, most reports have been on ovarian tissue freezing in pediatric FP, with only a few reports on OC or sperm freezing, as was the case in this study (17). Pediatric FP, whether OTC or OC, shows many scientific and medical differences from adult FP. The scientific aspects have been partially clarified with the gradual accumulation of knowledge about FP in children, but many years will be required to demonstrate its efficacy. Regarding medical care, pediatric patients reportedly often have systemic diseases, making OTC difficult to implement. Therefore, risk management at the time of surgery, ingenuity in surgical techniques, and cooperation among clinical departments have been shown to be necessary for success (18, 19). Particular attention should also be paid to ethical considerations, and due consideration of psychological issues is extremely important (14, 20). This study focused on and investigated psychological aspects of decision making for children considering FP, because the stress and fear of considering FP while battling a disease is likely to be much greater than that in adults. This is compounded by a lack of understanding or misunderstanding of FP due to the young age of the patients, underlying disease, and inadequate explanations from medical staff.

The results of this study showed that even minors who have not faced pregnancy, childbirth, or marriage have a strong desire to have children in the future (Q3). However, the lower the age, the more often the answer was "I don't know yet". Previous studies have reported that children over 12 years old have a strong desire to have genetic children, consistent with the present results (21). However, such results should be interpreted with caution as parental interventions cannot be ignored as a limitation of this study. In addition, in Q4 and Q5, a question was asked about the treatment being planned, and the proportion of those answering, "Know well" or "Know" increased with age. However, whether children understand correctly and how much detail they know has not been verified, so caution is also required regarding the interpretation of this result. Question 6 asked about the risk of impairing fertility during treatment of the underlying disease. This was a question that could be psychologically stressful to children. As expected, the ratio of "Know well" and "Know" was high and increased with age. This indicates the possibility that the content of information provided by doctors who treat primary diseases changes according to age. Similarly, a tendency was seen for an increasing understanding of the reason for visiting the FP hospital and knowledge about FP with increasing age. However, when asked if they would like to hear an explanation about FP, a certain proportion of respondents answered "Don't mind". In fact, some children may not have been positive about FP. As a post-explanation questionnaire, questions were first asked about the effect of treatment for the primary disease on fertility and the understanding of FP treatment. The survey with post-Q1 and -Q2 found no answers of "Don't really understand" or "Don't understand at all", suggesting that basic understanding was relatively high in all age groups, showing the validity of providing explanations regarding FP. As in Q4 and Q5 above, this result has not been objectively confirmed. Therefore, one limitation is that the level of actual comprehension remains unclear. In addition, most children had not undergone objective evaluation of intelligence. Some diseases, such as brain tumors, cause developmental problems, and in this study only one patient with brain tumor (10 years old) had been tested for intelligence (Wechsler Intelligence Scale for Children, fourth edition). Intellectual development is an important factor that influences the significance of FP, and cannot be ignored, especially regarding FP for children. After the explanation of FP, willingness to receive FP was confirmed in post-Q3. Almost all children showed a willingness to undergo FP. The reason why children who were scheduled to undergo OTC also responded to OC was that the combined procedure was explained (post-Q4). In addition, since some respondents answered "Don't really understand" regardless of age, tools to promote better understanding seem desirable. In any case, the majority of positive responses were attributed to the participation of the child in their own decision-making (21–23). In addition to pain and fear, cost concerns were raised as an issue for FP (post-Q5). Economic problems have been reported as a typical barrier for adults (24, 25) and this result is very important, since children can struggle with financial problems for FP in the same manner as adults. In addition, in post-Q6, -Q7, and -Q8, the lack of negative reactions to the explanations about FP indicates that direct explanation about FP to children is not harmful, regardless of whether they undergo FP. This was attributed to the participation of the child in the decision-making process, as described above. However, in post-Q8, some children (ages 6 and 11) said they did not really understand, indicating a need to develop even higher quality explanation methods. Finally, as mentioned above, the results of this survey cannot deny the influence of parental intervention. In other words, it is possible that the child gave the answer that the parent wanted. Of course, we asked the parents not to impose their opinions on the

children, and basically only asked them to explain the questions. Therefore, it is presumed that the patient's intention is basically reflected. However, it was not possible to exclude completely the involvement of parents due to issues of ethics and comprehension, but in the future, it would be desirable to plan a survey that children can complete by themselves using devices such as tablets using age-appropriate animation. Such developmental research will reveal actual children's perceptions, understandings, and feelings. In addition, parental validation is important in research on FP for children. In terms of comprehension, perception, and attitude, I think we should also test our parents. Also, it is also necessary to collect more cases for boys. It is necessary to consider the differences between boys and girls in understanding and attitudes regarding fertility.

5 Conclusion

Categorically determining the lower age and intelligence limits at which explanations of FP can be understood is difficult. One challenge of pediatric FP is precisely the need to tailor explanations of procedures according to the understanding of the individual child.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Please contact Nao Suzuki, nao@marianna-u.ac.jp.

Ethics statement

The studies involving human participants were reviewed and approved by institutional review board at St. Marianna University School of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

ST drafted the manuscript. ST, SF, MS, YY, TM, HK and NS designed the research and contributed to the critical discussion. ST, YI, YoS, HI, RK, ES, KI, YuS, KT, KOy, DK, KN, KOd, YH, LM, AI, AF, LA contributed to collecting and analyzing data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Trends and outcomes of fertility preservation for girls, adolescents and young adults with Turner syndrome: A prospective cohort study

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Background: In Scandinavian countries, programs for fertility preservation (FP) are offered free of charge at tertiary-care university hospitals to all patients facing infertility risks due to malignant diagnoses or benign conditions. In this prospective study we aimed to investigate trends and outcomes of FP indicated by a diagnosis of Turner syndrome.

Methods: Prospective cohort study of patients with Turner karyotype receiving fertility preservation counselling at the Karolinska University Hospital between 1 January 1999 and 31 December 2021.

Results: The cohort included 100 women and girls that received counselling, whereof 27% were prepubertal girls, 59% were adolescents and 14% of adult age. Before 2006 all patients were referred for fertility counselling at the time of Turner diagnosis. Based on updated guidelines, mainly patients who showed signs of puberty were referred after 2006. As a result, spontaneous menarche was more common in the later period. In total, 39% of the cohort had monosomal karyotype (45X), 20% had 45X/46XX or 45X/47XXX mosaicisms and 36% had an X-chromosomal structural anomaly. Ovarian tissue cryopreservation was planned for 73% of all patients, and oocyte cryopreservation following gonadotropin stimulation was planned for 10% of the patients. Follicles were present in 25% of all biopsies analyzed. Adolescents were more likely to have follicles present (30%) than prepubertal girls (16%) or adult women (17%). The ten patients that underwent gonadotropin stimulation for oocyte cryopreservation underwent a total of 15 cycles and eight patients successfully preserved oocytes. In total, 26% of the cohort has undergone fertility treatment or expressed further interest in fertility preservation. Six women have given birth using donated oocytes and three following spontaneous conception. Two women have undergone re-transplantation of cryopreserved ovarian tissue, without regaining ovarian function, and none of the women that have cryopreserved oocytes has returned to use them.

Conclusion: Fertility counselling for girls with Turner syndrome should ideally be offered at onset of spontaneous puberty to improve the chances of fertility preservation. Since the girls and women in this cohort are still young, the return rate and utilization of the preserved tissue and oocytes is expected to increase with time.

Clinical Trial Registration: ClinicalTrials.gov, identifier NTC04602962.

KEYWORDS

fertility preservation, Turner syndrome, fertility, ovarian tissue cryopreservation, pre-pubertal girls, adolescents

Introduction

Turner syndrome is the most common sex chromosome abnormality in women, with a prevalence of 1/2500. Diagnosis is most common in early childhood or adolescence, but in some cases the syndrome is diagnosed later in life, often related to an infertility work-up. The syndrome has a wide phenotypic spectrum caused by complete or partial absence of an X chromosome. Due to the range of possible abnormalities, including mosaicisms, Turner syndrome does not always present phenotypically and may remain undiagnosed throughout life (1). However, as widely acknowledged, Turner syndrome has a direct negative effect on fertility and most girls diagnosed with Turner syndrome will not undergo spontaneous puberty. It is estimated that approximately 20% of the girls diagnosed with Turner syndrome will spontaneously present initial puberty development, usually breast development, but only 16% of the girls will proceed to menarche (2). The likelihood to present with clinically evident ovarian function is higher in patients with X-chromosome mosaicisms (45X/46XX, 45X/47XXX) than in those having other X-chromosome variants (46X with ring chromosome, 46X with deletion, 46X with isochromosome, or 45X monosomy) (3). Girls with diagnosed Turner syndrome who show signs of ovarian function in childhood and early adolescence most often develop premature ovarian failure around the time of puberty due to rapid atresia of the follicles (4). It is currently recommended to offer reproductive counselling when a Turner diagnosis is confirmed and also to perform a careful cardiac evaluation to exclude comorbidities (5–7).

In women with Turner syndrome cardiac features may contraindicate a future pregnancy. Congenital heart abnormalities and aortic dilatation, which are strongly associated with life-threatening aortic dissection, are more common in Turner patients, where the incidence of maternal and postpartum death has been reported as high as 2% (8–10). If congenital heart abnormalities or acquired aortic dilation is present in women with Turner syndrome, medical assistance to achieve pregnancy is not recommended and other options for parenthood should be advised (5). While data are still heterogeneous, retrospective studies have indicated that with adherence to health care guidelines pregnancies can proceed without increase in cardiovascular complications (8–14).

In Sweden, a National Healthcare Program for patients with Turner diagnosis has been established since 1994, updated subsequently in 2000 and 2013, and is currently known as the Swedish Turner Academy. The program includes recommendations on how girls and women with Turner syndrome should be followed-up throughout life by multidisciplinary teams at the Turner centers established at all university hospitals (15). Since 2013, the program recommends that discussions regarding reproductive options should be initiated soon after diagnosis, and routinely revisited at the time of transition from pediatric to adult healthcare. In all patients that receive a Turner diagnosis irrespective of age, a thorough cardiac evaluation is performed with echocardiography and/or magnetic resonance imaging. This way the cardiovascular status is known before fertility preservation discussions would take place (5).

In order to preserve fertility in patients with Turner syndrome, fertility preservation (FP) should be offered at an early age, before oocyte depletion (6, 16, 17). However, there are no reliable methods to predict the progress of atresia, nor can it be determined if the follicles in pre-pubertal girls are functional or not. This makes routine implementation of FP difficult in patients with Turner syndrome. In most FP programs it is established that if a girl reaches spontaneous menarche, ovarian stimulation and collection of oocytes can be considered (18, 19). In young pre-pubertal girls, it is more difficult to establish an optimal method for FP. A previous Swedish study reporting on ovarian biopsies and ovarian tissue cryopreservation in Turner patients revealed that the probability of identifying ovarian follicles in the biopsies increased if the Turner karyotype showed a mosaicism, if the girls had developed spontaneous puberty, and if the serum Follicle-Stimulating Hormone (FSH) and Anti Müllerian Hormone (AMH) concentrations were normal for age (8). As shown in that study, biopsies were feasible in 47 of 57 girls and follicles were identified in 15 cases. Finding follicles in the ovarian biopsies supports future fertility treatment, e.g., by re-transplantation of the tissue, or, if ovarian function is evident during adolescence or early adulthood, by ovarian stimulation as a complementary option to girls who have previously cryopreserved ovarian tissue (16). A reason to offer

ovarian stimulation is the high efficacy of vitrified oocytes, while re-transplantation of ovarian tissue from women and girls with a reduced ovarian reserve at time of cryopreservation is expected to have low success rate.

At Karolinska University Hospital in Stockholm, fertility counselling and FP have been offered to girls and women with the Turner syndrome as part of a clinical study at the Karolinska University Hospital in Stockholm (3), and the center has also been the national reference center for fertility preservation for children. In this study we present treatment outcomes and long-term follow-up of a large cohort of women and girls with Turner syndrome.

Material and methods

The study cohort included all girls and women presenting with Turner syndrome who were referred for fertility preservation counselling at the Karolinska University Hospital, Section of Reproductive Medicine, in Stockholm, Sweden between 1 January 1999 and 31 December 2021. Data on referral, clinical characteristics and utilization of cryopreserved oocytes and tissues have been collected prospectively.

Counselling of girls and teenagers

According to the recommendation of the Swedish Turner multidisciplinary program, adolescent girls who present with spontaneous start of puberty should be referred for appropriate counselling on fertility preservation, and if possible, individualized fertility preservation (15). Nevertheless, no girls referred to the clinic have been excluded from the study, independently of age or pubertal development. Over the years, women and adolescent girls have been predominantly counselled towards clinically established methods. However, the methods for fertility preservation have improved over time as have the methods to determine the female ovarian reserve. Routine hormonal measurements started to be implemented at our center around 2006 using validated clinical methods at Karolinska University Laboratory. Earlier measurements were executed at either the Research Laboratory for Women's Health, Karolinska Institutet (FSH, LH, AMH) or the Central Laboratory for Clinical Chemistry, Karolinska University Hospital (FSH) as previously described by Borgström et al., 2009 (3). As most patients before 2006 were referred for fertility preservation regardless of hormonal status, we have divided the cohort over time before and after 2006.

In most cases, counselling was provided to the patients and their families by a pediatrician and also a reproductive medicine specialist. Written age-adapted information, in two versions (for children and adolescents) was provided. Parents of minors were asked to sign an informed consent form; when teenagers were counselled and they assented, both the parents and the child signed the form. Counselling also included information on alternatives to becoming a parent, such as using egg donation or adoption. The possibility to undergo fertility preservation at a later stage was also discussed, in case the patients elected not to undergo FP at time of referral (20).

At the time of counselling, if deemed appropriate and if the patients agreed to these examinations, ovarian reserve was evaluated by counting antral follicles through transvaginal ultrasound and by measuring serum concentrations of anti-Müllerian hormone. In several cases the results of the exams were also used to evaluate the long-term benefit of undergoing FP.

Girls and teenagers were most often counselled to laparoscopic retrieval of ovarian tissue for cryopreservation, where the need of future re-transplantation through additional surgeries in order to regain tissue functionality and fertility was explained. Patients were also informed on the possible advances in methods for *in vitro* tissue culturing of follicles to mature oocytes. The amount of ovarian tissue retrieved was individualized in all cases and discussed with the patients. After 2009 stimulation for oocyte cryopreservation through vitrification was also offered as an option to adolescent patients, if the girls had developed puberty and proceeded through menarche and wished to undergo the procedure, which includes monitoring with transvaginal ultrasound examinations and transvaginal follicular aspiration (19). The expected efficacy of the FP methods according to the current state of knowledge was explained.

Ethical approval for the study was granted by the Ethical Review Board of Karolinska University Hospital (Dnr 427/03) and the Regional Ethics Committee of Stockholm (Dnr 2011/1158-31/2, 2014/470-32, 2016/2530-32 and 2018/2255-32). Written informed consent to participate in this study was provided by the participants, or by the participants' legal guardian/next of kin.

Results

In total, 100 patients with Turner karyotype were counselled for fertility preservation between 1999 and 2021. The majority (59%) of the patients were between 13 and 17 years of age, 27% were prepubertal and 14% were adults. More than half (65%) the patients were referred for fertility counselling between 1999-2005 (Table 1).

Of all patients with Turner karyotype, 39% had monosomal karyotype (45X), 20% had 45X/46XX or 45X/47XXX mosaicism and 36% had an X-chromosome structural anomaly. After 2006 only 20% of the referred girls had a 45X monosomy (Table 1).

Fertility preservation methods applied

Ovarian tissue cryopreservation was planned for 74% of all patients; 89% of the prepubertal girls, 71% of adolescents and 54% of adult women. Oocyte cryopreservation was planned for 10% of adolescents and 15% of adults. In total, 18% of the patients did not undergo any fertility preservation treatment; 11% of girls, 19% of adolescents and 31% of adult women. Two patients who cryopreserved tissue also returned to cryopreserve oocytes later (Table 2).

Among the 73 patients with Turner karyotype who were planned for ovarian tissue biopsy, no ovarian pathologies were detected for 51% of patients, streak ovaries were found in 42% and 7% had no ovaries

TABLE 1 Description of the cohort by calendar period of referral.

	1999-2005 N=65	2006-2021 N=35	All patients N=100
Age - Mean ± SD	13.8 ± 0.35	14.5 ± 0.84	14.0 ± 0.4
Age group			
Girls (1-12y)	21 (32%)	6 (17%)	27 (27%)
Adolescents (13-17y)	37 (57%)	22 (63%)	59 (59%)
Women (18-27y)	7 (11%)	7 (20%)	14 (14%)
Turner Syndrome karyotype			
Monosomy 45X	32 (49%)	7 (20%)	39 (39%)
Mosaic 45X/46XX, 45X/47XXX	8 (12%)	12 (34%)	20 (20%)
X-chromosome structural anomaly[a]	23 (35%)	23 (35%)	36 (36%)
Missing	2 (3%)	3 (9%)	5 (5%)
Spontaneous start of puberty			
Not yet (at time of counselling)	9 (14%)	5 (14%)	14 (15%)
Yes	19 (29%)	26 (74%)	45 (45%)
No	32 (49%)	4 (11%)	36 (36%)
Missing	5 (8%)	0 (0%)	5 (5%)
Tanner stage (after puberty)			
2	3 (16%)	0 (0%)	3 (7%)
3	2 (11%)	1 (4%)	3 (7%)
4	3 (16%)	3 (12%)	6 (13%)
5	11 (58%)	21 (81%)	32 (71%)
Missing	0 (0%)	1 (4%)	1 (2%)
Spontaneous menarche (after puberty)			
Yes	13 (68%)	26 (100%)	39 (87%)
No	6 (32%)	0 (0%)	6 (13%)
Fertility preservation planned			
None	2 (3%)	16 (46%)	18 (18%)
Ovarian tissue cryopreservation[b]	63 (97%)	10 (29%)	73 (73%)
Oocyte cryopreservation	0 (0%)	9 (26%)	9 (9%)

[a]Includes isochromosome, ring chromosome, deletions and translocations of X, or Y-fragment.
[b]One patient who cryopreserved ovarian tissue at ages 12 returned for oocyte cryopreservation at age 16, only counted as ovarian tissue cryopreservation in the table.

(Table 2). In a minority of patients, the uterus was either small (3%) or non visualized (11%). In 11 cases, no ovarian tissue could be retrieved due to absent ovaries or streak ovaries observed during the laparoscopic procedure. In total, 85% of the patients had ovarian cortical tissue cryopreserved (Table 2).

Between 1999 and 2005, 65 girls were referred for fertility preservation, 57 of them as part of a study to evaluate fertility potential in Turner patients through measurements of fertility markers and through follicle counts in biopsied tissue (3). Among these 65 girls, 29% had spontaneous start of puberty and 97% were planned for ovarian tissue cryopreservation (Table 1). Follicles were found in 15 of the 57 biopsies analyzed (26%). In these 15 biopsies, the number of follicles varied from 0.7–1200/mm^3. All but one patient with streak ovaries did not have observable follicles. Adolescents were more likely to have follicles present (30%) than girls (16%) and adult women (17%) (Table 2).

Since 2006, 35 Turner patients have been referred for fertility counselling. 26 (74%) patients had both spontaneously entered puberty and had menarche, 5 (14%) had not yet entered puberty but were below the age of twelve, and 4 (11%) had not entered puberty. Among them 19 (54%) proceeded with fertility preservation measures (Table 1). 10 (29%) underwent biopsies for ovarian tissue cryopreservation and 10 (29%) were stimulated for oocyte cryopreservation (Table 2). All patients undergoing oocyte

TABLE 2 Ovarian tissue biopsy by year of referral.

	1999-2005 N=63	2006-2021 N=10	All patients N=73
Age group			
Girls (1-12y)	21 (33%)	3 (30%)	24 (33%)
Adolescents (13-17y)	35 (56%)	7 (70%)	42 (58%)
Women (18-27y)	7 (11%)	0 (0%)	7 (10%)
Ovaries			
No abnormalities detected	28 (44%)	9 (90%)	37 (51%)
Streak ovaries	30 (48%)	1 (10%)	31 (42%)
Ovaries absent	5 (8%)	0 (0%)	5 (7%)
Uterus			
No abnormalities detected	53 (84%)	10 (100%)	63 (86%)
Small uterus	2 (3%)	0 (0%)	2 (3%)
Uterus non visualized	8 (13%)	0 (0%)	8 (11%)
Biopsy taken			
Yes	52 (83%)	10 (100%)	62 (85%)
No	11 (17%)	0 (0%)	11 (15%)
Follicles in biopsy			
Not analyzed	6 (10%)	10 (100%)	16 (22%)
No	42 (67%)	0 (0%)	42 (58%)
Yes	15 (23%)	0 (0%)	15 (21%)

cryopreservation had spontaneously entered puberty and also had spontaneous menarche. Among the 16 (46%) that have not yet done any FP measures, five (28%, currently at a mean age of 14) are planned for follow up, five (28%, with a mean age of 17 at counselling) had spontaneous puberty and menarche with hormonal levels indicating remaining fertility but are currently not planned for fertility preservation, six (35%, with a mean age of 19 at counselling) had low hormonal levels and are not planned for fertility preservation, among them one diseased. Among the six with low hormonal levels three had a monosomy.

The 10 patients that proceeded with oocyte cryopreservation, underwent a total of 15 cycles of controlled ovarian stimulation. 8 patients successfully cryopreserved oocytes. The mean age at stimulation was 17.2 years. In the seven patients with AMH measurements, the mean level was 1.1 µg/l. The mean number of mature oocytes cryopreserved in each stimulation cycle was 5.1 (range 0-19) (Table 3). The oocytes were cryopreserved using vitrification.

Fertility treatments and follow-up

At the end of follow-up, 14 girls and adolescents were below 20 years of age, 41 women were in their 20's and 42 in their 30's. Three patients were lost to follow up; one deceased and two emigrated. A total of 26 adolescents and women had documented contact with healthcare concerning their fertility, and 20 returned to the reproductive medicine unit. Eight girls and women returned for further fertility counselling, three women underwent a new fertility preservation and seven women elected fertility treatments using egg donation. To date, six women have given birth following fertility treatment using donated oocytes, whereof two had treatments abroad. Three women have reportedly had children following spontaneous conception; one woman with X-chromosome

TABLE 3 Oocyte cryopreservation.

	Adolescents and women N=10 **
Age, y	17.2 ± 2.6 (14–22)
AMH, µg/l	1.1 ± 1.0 (0.3-3.2)
Number of stimulations, n	1.5 ± 0.5 (1–2)
Oocytes cryopreserved* per cycle, n	5.1 ± 5.4 (0-19)
Oocytes cryopreserved* per patient, n	7.4 ± 7.0 (0-19)

Data from patients referred for Fertility Preservation (FP) at Karolinska University Hospital between 1998 and 2021, who underwent controlled hormonal stimulation for oocyte cryopreservation. Numbers presented are mean ± SD (range). The stimulation cycles were performed 2011-2022 and all oocytes were cryopreserved using vitrification. AMH, Anti-Müllerian hormone.
*Information on number of oocytes missing for one patient, no oocytes could be cryopreserved for two patients.
** 5 patients underwent repeated treatments.

structural anomaly, one woman with mosaicism and one woman with monosomy. No births following the use of stored oocytes have been achieved so far.

Ovarian tissue transplantation

Two women in this cohort have undergone re-transplantation of cryopreserved ovarian tissue. In one case the karyotype showed X-chromosome structural anomaly and in the other case a mosaicism. None of the women have regained ovarian functionality as measured by repeated blood samples estimating levels of hormone secretion (Serum Estradiol, AMH, FSH, LH, Progesterone).

Discussion

This unique cohort provides insight into the fertility choices and options available for young girls with Turner syndrome. In Sweden fertility counselling and fertility preservation is tax-funded and girls and women with Turner syndrome are currently followed up throughout life by multidisciplinary teams at Turner centers established at all university hospitals according to the National Healthcare Program for Turner syndrome. Reproductive options are discussed soon after diagnosis and also at the transition from pediatric to adult healthcare. In presence of any congenital heart or vessel disease pregnancy is not recommended. The current recommendations are that a girl that has a spontaneous start of puberty should be referred for reproductive counselling, and if feasible, based on health and fertility status, stimulated for oocyte cryopreservation before her oocyte reserve is too much reduced by the rapid atresia.

This cohort has been assembled from the fertility preservation unit at Karolinska University Hospital over a period of 22 years. It should be noted that most of the patients who were included before 2006 (57 of 65) underwent ovarian tissue cryopreservation independent of age and pubertal status, as part of a study to evaluate the fertility potential of Turner girls (3). Based on the results from that study, as well as national and international guidelines, most girls included from 2006 and onwards have been referred for fertility preservation only at signs of spontaneous puberty or at expected start of puberty due to a karyotype with mosaicism. When possible, oocyte cryopreservation has been the fertility preservation method of choice, as the method gained recognition as a clinically established method earlier than ovarian tissue cryopreservation, which was still considered experimental until very recently (21).

A majority of the counselled patients underwent fertility preservation, most often through ovarian tissue cryopreservation. Nearly half the patients who underwent a tissue biopsy had observed abnormalities of the ovaries and one in ten patients had no uterus. In total 11 laparoscopies to obtain ovarian biopsies had to be terminated without tissue retrieval. Following the implementation of stricter criteria for referral, all planned biopsies could proceed successfully.

The implementation of more accurate methods of estimating the female ovarian reserve, such as using biochemical markers including serum AMH, and the adherence to updated guidelines drastically reduced the referral rate and only 35 girls and women with Turner syndrome have been counselled between 2006 and 2020. In this later cohort, 74% had spontaneous menarche, ten patients have cryopreserved ovarian tissue, eight patients have undergone successful oocyte cryopreservation, most in late adolescence, and six are planned for follow-up. Only six patients referred after 2006 have been counselled to proceed with other options for family planning due to low hormonal levels or other health issues. This can be compared to the 65 girls counselled between 1999 and 2005 where 71% did not have signs of spontaneous puberty, no ovarian stimulations for oocyte cryopreservation were performed and 97% of patients were planned for ovarian tissue cryopreservation, whereof 15 patients (24%) had observable follicles the in biopsied tissue.

The live birth rate after re-transplantation of cryopreserved ovarian tissue has been estimated to 33-38% in several other patient groups (22–24). However, the potential for pregnancy and live birth is directly correlated with the number of functional primordial follicles available in the biopsied ovarian tissue (25). To date outcome data are limited when ovarian tissue cryopreservation is performed at a very young age (26–28). In patients with Turner syndrome, where rapid atresia is common, the success rate of ovarian tissue re-transplantation is uncertain. While age alone was previously shown as a non-significant factor in predicting the occurrence of follicles in girls above 12 years of age, it should be noted that these results were from a cohort where the patients where all below the age of 20 (3).

Six women in the cohort have given birth following assisted reproduction with donated oocytes. In addition, we have observed five spontaneous pregnancies followed by live births in three women from the cohort (3%), but cannot exclude the possibility of additional undocumented cases. A large French study by Bernard et al. (29) reported a live birth rate of 3.8% (18/480) and an overall 5.6% (27/480) prevalence of spontaneous pregnancies in women with Turner syndrome. Most of these pregnancies occurred in women with mosaic karyotype and only 0.4% (2/480) in women with a non-mosaic (45X) karyotype. Among the three women with children after spontaneous pregnancy in our cohort, one had a monosomy, one an X-chromosome structural anomaly and one a mosaicism. In a recent UK study including 127 pregnancies in 81 women with Turner syndrome (30), 58% (73/127) of pregnancies were spontaneously conceived and all others were conceived using oocyte donation. Among pregnancies in women with monosomy (45X), 29% (9/31) were spontaneously conceived. To our knowledge, there is only one report so far on a successful fertility treatment using cryopreserved oocytes in a woman with Turner syndrome (31).

This study has amassed a large prospective cohort of Turner patients but is limited by the lack of data on follicle status in the biopsies taken after 2006 and incomplete information on spontaneous births. Further, most patients in the cohort are still relatively young. The return rate in the cohort is expected to increase as all patients are still eligible for fertility preservation

and some are still prepubertal or adolescent. All patients that have proceeded with fertility treatments can be found among the 42 patients in their thirties. None of the patients that have preserved oocytes have yet reached 30 years of age.

We report two attempts at re-transplantation of ovarian tissue, but as of yet there has been no successful re-transplantation nor any use of the cryopreserved oocytes in our cohort. As the scarcity of promising follow-up data discourages routine use of early ovarian tissue cryopreservation, Turner girls with early onset atresia currently lack promising FP prospects. However, the implementation of the current guidelines with counselling and follow up from onset of puberty has proven useful for identifying Turner girls eligible for fertility preservation.

Data availability statement

The datasets presented in this article are not readily available because the datasets could possibly identify the individual participants due to the limited cohort size. Requests to access the datasets should be directed to kenny.rodriguez-wallberg@ki.se.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Review Board of Karolinska University Hospital (Dnr 427/03) and the Regional Ethics Committee of Stockholm (Dnr 2011/1158-31/2, 2014/470-32, 2016/2530-32 and 2018/2255-32). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Conceptualization, KR-W. Methodology, KR-W and FL. Formal Analysis, FL. Data Curation, KR-W and FL. Writing – Original Draft Preparation, KR-W, HN and FL. Writing – Review & Editing, KR-W, FS, HN, FL. Funding Acquisition, KR-W. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Ovarian tissue cryopreservation in the pediatric with rare diseases- experience from China's first and the largest ovarian tissue cryobank

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Background: There is limited information about the efficacy of ovarian tissue cryopreservation (OTC) in children. In the present study, we report eight patients with rare diseases who underwent OTC in China's first and largest ovarian tissue cryobank.

Procedure: Data from girls with rare diseases who underwent OTC between September 2020 and November 2022 were retrospectively analyzed. We also compared the number of cryopreserved cortex pieces, follicle number, and AMH in those with rare diseases and age-matched children with non-rare diseases who also underwent OTC in our cryobank.

Results: The median age of the children was 5.88 ± 3.52 (range 2-13) years old. Unilateral oophorectomy was undertaken *via* laparoscopy in all of the children. The diseases in the 8 patients were: 4 mucopolysaccharidoses (MPS I two cases, IVA two cases), 1 Diamond-Blackfan anemia (DBA), 1 Fanconi anemia (FA), 1 hyperimmunoglobulin E syndrome (HIES), 1 Niemann-Pick disease. The number of cryopreserved cortex pieces was 17.13 ± 6.36, and the follicle count per 2 mm biopsy was 447.38 ± 524.35. No significant difference in age, the count of cryopreserved cortex pieces, follicle number per 2 mm biopsy, and AMH level was seen between the 20 children with non-rare diseases and those with rare diseases.

Conclusions: The reports help practitioners counsel girls with rare diseases about fertility preservation. The demand for OTC in pediatrics will likely grow as a standard of care.

KEYWORDS

ovarian tissue cryopreservation, children, fertility preservation, rare diseases, hematopoietic stem cell therapy

1 Introduction

Diseases with an incidence of fewer than 1/10,000 newborns, a prevalence of less than 1/10,000, and a number of patients less than 140,000 are classified as rare diseases (RD). As of February 2022, there are more than 7,000 known rare diseases worldwide, accounting for about 10% of all human diseases. About 72%-80% of rare diseases are caused by structural changes or abnormal regulation of genetic material (1). China has over 20 million rare disease patients, with over 200,000 new patients yearly (2). Around the world, the treatment of rare diseases is probably the most significant medical challenge facing humanity today. Treatment of some rare diseases requires hematopoietic stem cell therapy (HSCT) (3). Advances in HSCT technology and supportive care have increased the number of long-term survivors (4), so fertility preservation (FP) for these patients is also essential.

Ovarian tissue cryopreservation (OTC) is the only FP method for prepubertal girls (5). OTC includes laparoscopic surgery to remove part or the whole ovary, followed by transport to the ovarian tissue cryobank for cryopreservation and storage (6, 7). Professional institutions must have the necessary equipment, trained personnel, and sufficient frozen stock to conduct OTC safely and with consistent quality. More than 200 babies worldwide have been born through this technique, including reports of frozen ovarian tissue collected before puberty and frozen-thawed ovarian tissue transplantation (OTT) after puberty (8–10). In 2019, the American Society for Reproductive Medicine (ASRM) recommended that OTC technology no longer be considered experimental (11). Minimal complications have been reported after laparoscopic surgery (12).

The Oncofertility Consortium's National Physicians' Cooperative (ON-NPC) published its experience with OTC in 114 girls<15 years old in 2018 (13). Germany's UniCareD cryobank stored frozen ovarian tissue from 104 girls with a mean age of 14 years between 2018 and May 2022 (14). Our center has cryopreserved ovarian tissue from more than 50 children (12).

Studies on FP in RD are limited. Because of the high incidence of POI for patients with genetic abnormalities, such as galactosemia and Turner syndrome (TS), experts recommend starting FP as soon as possible (15–19). FP counseling is also needed for children with RD who plan to undergo HSCT. This paper reports on the cryopreservation of ovarian tissue in 8 patients with RD to give medical workers more information and confidence and introduce patients to the FP center for consultation. A multidisciplinary team should always be involved in treating and managing RD patients.

2 Methods

2.1 Ethics statement

The Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University, approved the provision of centralized OTC (2017-KY-020-01; March 15, 2017). Ovarian tissue was received from different hospitals and transported to the centralized cryobank. The parents of each patient signed an agreement and informed consent for their child.

2.2 Ovarian tissue retrieval, transportation, and preparation

Eight girls with RD underwent OTC in our cryobank between September 2020 and November 2022 (mean ± SD, range, 5.88 ± 3.52 years, 2-13 years). Unilateral oophorectomy was undertaken *via* laparoscopy in the eight children. No complications were reported after laparoscopic surgery in the eight children.

After retrieval, the ovarian tissue was immediately put into the cooled Custodiol (HTK). The temperature was maintained at 4-8 °C during ovarian tissue transportation. The mean temperature on reaching the cryobank was 5.44 °C, and the transport time was no more than 12 hours. In a sterile laminar flow cabinet, ovarian tissue was prepared at HTK solution, maintained at 4 °C. The ovarian cortex was handled to be 1 mm thick, cut to 6 mm x 3 mm slices, and cryopreserved. After slow programmed freezing, the tubes were stored in a liquid nitrogen tank with a gas phase. In ovarian tissue preparation, standardized cortical biopsies with a diameter of 2 mm from different areas were evaluated for follicle density and viability assay. The procedures were according to the previous publications (12, 20).

Twenty age-matched children who underwent OTC because of non-rare diseases were selected to compare the number of cryopreserved ovarian cortex pieces, follicle number per 2 mm biopsy, and the level of AMH between patients with RD and those with non-rare diseases. They did not undergo gonadotoxic treatment before OTC.

2.3 Hormone levels before OTC

The levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and AMH in serum before OTC were evaluated. The details can be seen in our previous publication (15).

2.4 Analysis of follicle density

The number of surviving resting follicles was evaluated in biopsies by Calcein-AM assay, which method has been described in the previous publication (21).

2.5 Statistical analyses

SPSS 22.0 was applied for analysis. The data following normal distribution were expressed by "mean ± Standard deviation (SD)"; otherwise, "mean ± Standard Error of Mean (SEM)". The differences between groups were compared by independent sample t-test. $P < 0.05$ indicates that the difference was statistically significant.

3 Results

3.1 Characteristics

3.1.1 Ages and diseases diagnosis

Characteristics of the 8 girls with OTC are shown in Table 1. The age of the 8 children was 5.88 ± 3.52 years old, range 2 to 13 years. The diseases in the 8 patients were: 4 mucopolysaccharidoses (MPS I two cases, IVA two cases), 1 Diamond-Blackfan Aanemia (DBA), 1 Fanconi anemia (FA), 1 hyperimmunoglobulin E syndrome (HIES), 1 Niemann-Pick disease. Ovarian tissue was cryopreserved because of planned HSCT.

3.1.2 Ovarian tissue retrieval, transportation, cryopreservation, follicle number, FSH, LH, and AMH

Unilateral oophorectomy was undertaken *via* laparoscopy in the children. The temperature during transport to a centralized cryobank was 5.44 ± 0.96°C. The number of cryopreserved cortex pieces was 17.13 ± 6.36 (mean ± SD), the follicle number per 2 mm biopsy was 447.38 ± 524.35 (mean ± SD) (Figure 1). The FSH was 3.46 ± 2.14 IU/L (mean ± SD), LH was 0.55 ± 0.53 IU/L (mean ± SEM), and AMH was 1.60 ± 1.21 ng/ml (mean ± SD).

3.2 The comparisons between rare diseases and non-rare diseases

In Figure 2, no significant difference in age, the number of cryopreserved cortex pieces, follicle number per 2 mm biopsy, and AMH level between 20 children with non-rare diseases and those with rare diseases (mean ± SD, 7.05 ± 3.16 vs. 5.88 ± 3.52, P=0.396; mean ± SD, 20.70 ± 7.39 vs. 17.13 ± 6.36, P=0.241; mean ± SEM, 947.35 ± 200.30 vs. 447.38 ± 185.39, P=0.153; mean ± SEM, 2.15 ± 0.36 vs. 1.60 ± 0.43, P=0.616, respectively).

4 Discussion

No studies have described OTC for DBA, FA, MPS-I, MPS-IVA, HIES, and Niemann-Pick disease. Patients with these diseases required high-dose chemotherapy with alkylating agents and/or total body irradiation as pre-treatment for HSCT. Therefore, the present study includes important information for OTC in children with RD.

With the development of Assisted Reproductive Technology (ART), embryos that do not carry explicit disease-causing genes can be transferred back to the maternal uterine cavity after embryo preimplantation genetic testing (PGT) for families with evident genes for RD/genetic disorders. This is an essential technical tool for the primary prevention and control of congenital disabilities and can stop the transmission of RD/genetic disorders in the family from the source and produce healthy offspring (22). Secondary prevention is prenatal screening, which requires amniocentesis during pregnancy to ensure again that the fetus does not carry the disease-causing genes. Tertiary prevention is post-birth screening.

DBA is a rare congenital intrinsic erythroid hypoplasia, with 7 cases/million live births, acknowledged in 2005 as the first human ribosomopathy (23, 24). The median age of diagnosis is two to three months, with 95% of the DBA cases diagnosed before two years and 99% before five years of age (25, 26). HSCT is safe and efficient in DBA and should be considered if a matched sibling or unrelated donor is available (26, 27). FA is a challenging disease, and HSCT is the only curative therapy for the hematologic complications associated with this disease (28). The favorable mean overall survival was 80.9%, and event-free survival was 79.3% (29).

TABLE 1 Patient Characteristics before OTC.

Patients	Disease	Age at OTC	Transport temperature	Number of cryo-preserved cortex pieces	Follicle number per 2mm biopsy	FSH (IU/L) before OTC	LH (IU/L) before OTC	AMH (ng/ml) before OTC
Case 1	Diamond-Blackfan anemia	7	4.7	10	128	3.47	0.01	0.48
Case 2	Fanconi anemia	7	5.8	17	122	2.2	0	1.15
Case 3	Mucopolysaccharidosis I	5	7.1	21	229	–	–	2.65
Case 4	Mucopolysaccharidosis IVA	3	6.2	17	1581	4.31	0.02	1.31
Case 5	Mucopolysaccharidosis IVA	3	4.9	13	437	6.83	0	0.67
Case 6	Mucopolysaccharidosis I	7	4.0	20	851	1.22	0	4
Case 7	hyper Ig E syndrome	13	5.3	29	133	1.02	3.75	1.88
Case 8	Niemann-Pick disease	2	5.5	10	98	5.16	0.06	0.65

OTC, ovarian tissue cryopreservation; FSH, follicle-stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone.

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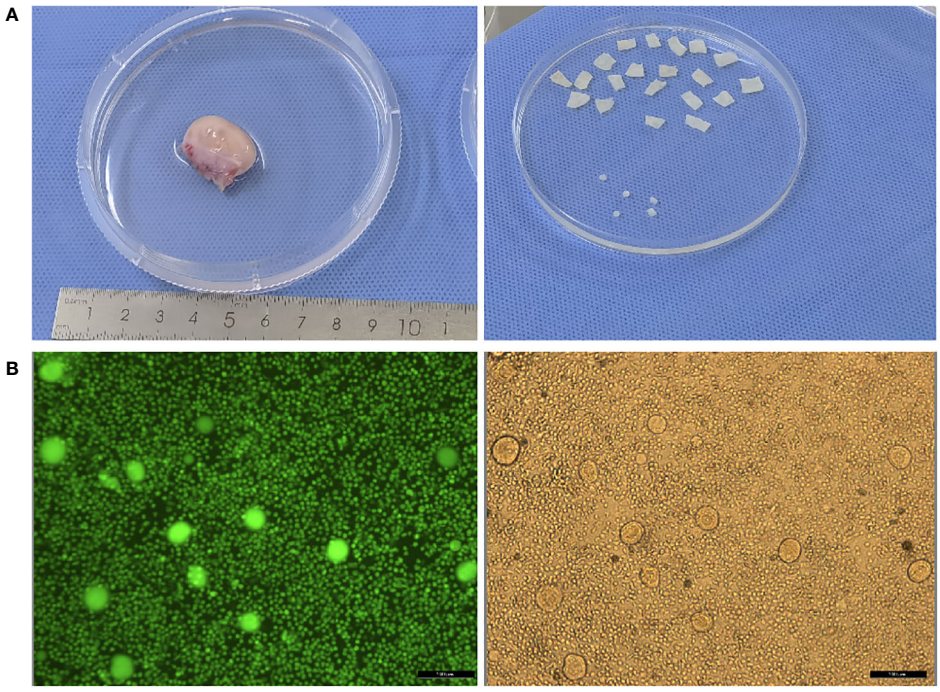

FIGURE 1
(A) Photos of ovary before and after ovarian tissue cortex preparation; **(B)** Detection of follicular activity in ovarian cortex.

MPS is a group of rare genetic diseases with abnormal glycosaminoglycan (GAG) catabolism (30). The overall incidence of MPS is estimated to be 1/100,000 live births, which varies according to region and race (31). HSCT has been used in patients with MPS I, II, IVA, VI, and VII, showing increased acceptance and therapeutic benefits (32). In 2011, a retrospective study evaluated the outcome of HSCT in 45 patients with MPS VI, with a 1-and 3-year survival rate of 66%. Patients who received HSCT had a longer life expectancy than those who did not receive treatment or enzyme replacement therapy (ERT) (33). The overall survival rate after transplantation was 90% (30). Standardized follow-up and a multidisciplinary team help accurately assess long-term post-transplantation outcomes and improve the quality of life (34).

HIES is primary immunodeficiency that results from heterozygous mutations in the signal transducer and activator of the transcription 3 genes. Some patients with HIES have been reported to be treated with HSCT. However, the efficacy of HSCT

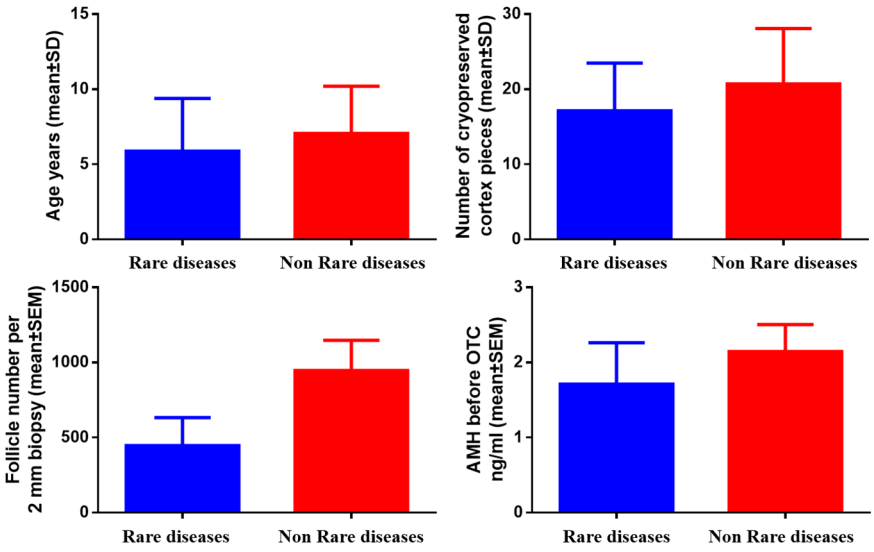

FIGURE 2
Comparison of age, number of cryopreserved cortex pieces, follicle number per 2 mm biopsy, and AMH in children with and without rare diseases.

for autosomal dominant HIES is inconsistent (35). HSCT potentially benefits the severe phenotype of Niemann-Pick disease patients (36). However, pre-treatment with radiotherapy and high-dose chemotherapy before HSCT can seriously harm the ovaries, and 70~100% of young females develop premature ovarian insufficiency (POI) (37, 38).

Our study found no significant difference in ovarian size, follicle number, and AMH level before OTC between children with rare diseases and age-matched children with non-rare diseases. However, it did not include children with Turner syndrome who have accelerated follicle depletion prior to puberty (39–41). Also, further evaluation is necessary after increasing the sample size. The measurement of AMH levels is controversial because the assessment of ovarian reserve in children and adolescents using AMH levels has limitations (9, 42).

Ovarian size varies with age and pubertal development. The prepubertal ovary is smaller than the reproductive ovary; therefore, the entire unilateral ovary is usually removed (43). Many pediatric surgeons and gynecologists perform unilateral laparoscopic oophorectomy, using an ultrasonic advanced energy device to segment the ovarian artery and mesovarium. No major surgical complications have been observed with this technique (44).

With increasing evidence of live births and recovery of endocrine function, a British Fertility Society guideline concluded that prepubertal girls should be considered for OTC (45, 46). OTC and transplantation are now the only FP option for prepubertal girls (47, 48).

Our center is the first and largest ovarian tissue cryobank in China. Nearly 500 cases of ovarian tissue have been successfully preserved, 10 cases have been successfully transplanted, and the ovarian function has recovered after OTC and OTT (49). One patient with MDS successfully conceived naturally and delivered a healthy baby girl after OTC and OTT (50). With the cooperation of pediatrics, OTC in children in our center has increased in the last two years (12).

The limitation of this study is the lack of data regarding outcomes such as later fertility following the use of cryopreserved ovarian tissues. This should be mentioned when counseling by practitioners. The outcome in these patients will hopefully be reported on long-term follow-up. It should stimulate further research within this challenging scientific field.

5 Conclusion

To conclude, reporting information helps practitioners counsel girls with RD about FP and the preservation of ovarian endocrine function supported by OTC. The demand for OTC in pediatrics will likely grow as a standard of care.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Beijing Obstetrics and Gynecology Hospital, Capital Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

All authors qualify for authorship by contributing substantially to this article. XR: project leader, and project supervisor, evaluated the ovarian reserve function of each child, supervised and guided ovarian tissue biopsy, transport, preparation, cryopreservation, follow-up, interpretation of results, and provided critical comments and revised the first draft. JC: article preparation, ovarian tissue transportation, preparation, and cryopreservation. JD, FJ, and MG: ovarian tissue preparation and cryopreservation. RJ, YRW, and LL: biopsied ovarian tissue. YJW, LJ, YY, YL, ZW, JM, and MZ: ovarian tissue transportation, AM: experimental supervision, interpretation of results, and article revision. All authors reviewed the article's final version and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fertility preservation in pediatric healthcare: a review

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Survival rates for children and adolescents diagnosed with malignancy have been steadily increasing due to advances in oncology treatments. These treatments can have a toxic effect on the gonads. Currently, oocyte and sperm cryopreservation are recognized as well-established and successful strategies for fertility preservation for pubertal patients, while the use of gonadotropin-releasing hormone agonists for ovarian protection is controversial. For prepubertal girls, ovarian tissue cryopreservation is the sole option. However, the endocrinological and reproductive outcomes after ovarian tissue transplantation are highly heterogeneous. On the other hand, immature testicular tissue cryopreservation remains the only alternative for prepubertal boys, yet it is still experimental. Although there are several published guidelines for navigating fertility preservation for pediatric and adolescent patients as well as transgender populations, it is still restricted in clinical practice. This review aims to discuss the indications and clinical outcomes of fertility preservation. We also discuss the probably effective and efficient workflow to facilitate fertility preservation.

KEYWORDS

fertility preservation, oocyte cryopreservation, ovarian tissue cryopreservation, testicular tissue cryopreservation, pediatric

Introduction

Long-term survival for children and adolescents diagnosed with malignancy has steadily increased and exceeded 80% over the past decade (1–3). As these cancer survivors reach adulthood, a substantial proportion of them experience infertility associated with previous gonadotoxic chemotherapy and/or radiotherapy (4, 5). In addition to oncology treatment, other non-oncological conditions and related therapy may raise fertility problems, including nephrotic syndrome (6), Turner Syndrome (7) and systemic lupus erythematosus (8). Currently, fertility issues have been increasingly recognized as a major concern for those newly diagnosed patients and their families (9, 10). Failing to achieve parenthood raises tremendous psychosocial stress on patients and their families and impairs their well-being. While scientists have established a range of methods for fertility preservation, including

embryo cryopreservation, gamete cryopreservation, and gonad tissue cryopreservation (11), the clinical practice is not as satisfactory as expected (12, 13). This review aims to discuss the indications, methods, and clinical outcomes of fertility preservation in the pediatric and adolescent populations. The potential effective and efficient workflow to facilitate fertility preservation is discussed as well.

Indications for fertility preservation in the pediatric and adolescent populations

Oncological causes

The incidence of pediatric and adolescent cancers is estimated to range between 50 to 200 cases per million children per year (14, 15) and more than 80% of these cancers are now potentially curable with current treatments (1, 2). Chemotherapy and radiotherapy in cancer treatments can lead to temporary, long-term and permanent gonadal toxicity, making fertility impairment another issue that distresses cancer survivors and their families (16). Alkylating agents are highly gonadotoxic and are associated with premature ovarian insufficiency (17, 18) and oligo- or azoospermia (19) depending on agent and dose (17, 20).

In females, these treatments substantially accelerate the activation and atresia of primordial follicles, leading to premature ovarian insufficiency (POI) and permanent amenorrhea (18, 21–24). Depending on agents and regimes, Impact on fertility may be broadly classified in low (<20%), medium (20-80%), or high (>80%) (25). A 13-fold increased chance of developing premature menopause was observed in a childhood cancer survivor study (26). Ovarian radiation, on the other hand, can cause 50% of follicle depletion at the dosage of 2 Gy and 60% chances of ovarian insufficiency at 2.5-5 Gy (25). Besides, long-term follow up of pediatric patients also demonstrated significant decline in anti-Müllerian hormone (AMH) after cancer therapy (23, 27), suggesting fertility losses and future fertility problems.

Spermatogenesis is particularly sensitive to chemotherapy and radiotherapy (28). Long-term follow up observed 25% and 28% of adult survivors of childhood cancer suffered from azoospermia and oligospermia after chemotherapy with cyclophosphamide equivalent dose less than 4000 mg/m^2 (29). In some regimes, several agents were administrated together, when cyclophosphamide is given > 7500 m g/m^2, almost all patients developed permanent azoospermia (16). Similarly, exposure to radiation causes germ cell loss in a dose-dependent manner (30), with immature spermatogonia the most radiosensitive, followed by spermatocyte and spermatid (28). Radiation at 0.1 Gy can result in morphological and quantitative changes to spermatogonia, increasing the dosage leads to spermatocyte and spermatid reduction (31). The threshold of radiation dose leading to permanent azoospermia remains unclear. But Castillo et al. found that all boys with acute lymphoblastic leukemia receiving testis radiotherapy at dose over 12 Gy developed azoospermia (19). A more recent study suggests that testicular radiation > 6 Gy may lead to permanent infertility (31).

Non-oncological causes

Younger patients affected by certain non-oncological medical conditions which require gonadotoxic treatments are potential candidates for fertility preservation as well (17, 32, 33). For example, gonadotoxic alkylating agents are widely used for diseases including nephrotic syndrome (6), systemic lupus erythematosus (34, 35), refractory idiopathic thrombocytopenic purpura (36). Also, a range of hematopoietic disorders, including thalassemia major, sickle cell anemia, aplastic anemia and myeloproliferative diseases, may be treated with hematopoietic stem cell transplant, which preconditions alkylating chemotherapy with or without radiotherapy (37, 38). In addition, some diseases can affect patients' fertility at an early age, including Turner syndrome (7, 39), Klinefelter's syndrome (40), fragile X syndrome (33), endometriosis (41), and gonad injury (42). Transgender populations receiving gender-affirming treatments may also require fertility preservation (43). Some of the most common non-oncological conditions which may require consideration of fertility preservation is presented in Table 1.

Potential risks for fertility impairment vary depending on patients' age, gender, body mass index, medical condition, and subsequent treatment scheme. A comprehensive and individual assessment is essential to determine the appropriate timing and methods for fertility preservation (46). Previous guidelines have extensively discussed the fertility risk assessment of specific agents

TABLE 1 Non-oncological indications for fertility preservation.

Conditions	Diseases
Autoimmune diseases (35, 44)	Systemic lupus erythematosus, Crohn's disease, Behcet's disease, Sjogren's syndrome, systemic scleroderma, nephrotic syndrome, multiple sclerosis, acute progressive nephritis syndrome, *etc.*
Hematopoietic stem cell transplantation (38)	β-thalassemia major, severe aplastic anemia, sickle-cell disease, Fanconi's anemia, *etc.*
Other conditions causing POI or spermatogenic failure	Turner syndrome (7), Klinefelter's syndrome (40), fragile X syndrome (33), endometriosis (41), ovarian/testicular torsion, benign ovarian tumors, galactosemia (45), gonad injury (42), *etc.*
Transgender populations (43)	Not applicable

POI, premature ovarian insufficiency.

or therapy regimes (25, 47–51), which have provided useful guidance to current practice.

Available options for fertility preservation

Females

Oocyte cryopreservation

Embryo cryopreservation, as a long-established fertility preservation method, can guarantee the best outcomes for fertility preservation. However, oocyte cryopreservation is preferred since most adolescents are unlikely to have a permanent partner and using donor sperm is less desired and poses ethical issues (11, 17). Since 2012, oocyte cryopreservation is no longer considered an experimental method for fertility preservation (52). However, outcomes in adolescents are less clear.

Controlled ovarian stimulation is the most effective strategy to obtain mature oocytes (53). However, conducting ovarian stimulation on a basis of diagnosed disease requires modifications to conventional protocols to address potential restrictions, including limited time allowed, and temporary exposure to high estradiol levels (54). Advances in ovarian stimulation have allowed fertility specialists to finish ovarian stimulation and oocyte retrieval within two weeks (55, 56). In urgent situations, the gonadotrophin-releasing hormone (GnRH) antagonist protocol is considered optimal for its short time and safety. Meanwhile, random and double stimulation are feasible alternatives (32, 57). In non-urgent situations, on the other hand, both GnRH antagonist protocol and long protocol are appropriate (32). Anti-estrogenic agents may be added to abolish estradiol reproduction in estradiol-sensitive diseases (53, 54). In addition, cryopreservation of *in vitro* maturated oocytes may be a feasible strategy when present with time constraints (58), which eliminates potential estrogen elevation and minimizes delay in treatment. Immature oocytes can be obtained at the time of ovarian tissue cryopreservation or oophorectomy as well (59). Various cryopreservation methods have been developed to freeze oocytes. If patients survive the original diseases and desire pregnancy in the future, these oocytes can be thawed and used for assisted reproductive techniques (Figure 1A). Recent advances in cryoprotectants, cryopreservation techniques (vitrification), and fertilization with intracytoplasmic sperm injection (ICSI) have significantly improved the clinical efficacy of cryopreserved oocytes (60–62). A series of studies which investigated the efficacy of different cryopreservation protocols concluded that vitrification outperforms slow freezing (63). The survival rate of vitrified oocytes ranges between 73.6% and 92.7%, significantly higher than that of slow freezing (58.0%-72.3%) (63–70). Vitrification is also superior regarding other outcomes like fertilization, implantation, clinical pregnancy, and live birth (63, 65, 71).

GnRH-agonist protection

The clinical efficacy of gonadotrophin-releasing hormone agonists (GnRH-a) during chemotherapy is controversial (54) and current recommendations regarding its use remain conflicting (11, 32, 53). Some meta-analyses evaluated the protective effect of GnRH-a during chemotherapy in premenopausal patients with breast cancer or lymphoma. Lower risks of chemotherapy-induced POI/amenorrhea and a higher number of spontaneous pregnancies after GnRH-a withdrawal were observed in the study group (72–75). However, the evidence is relatively weak due to heterogeneous populations, varying chemotherapy regimens and study endpoints (72, 76, 77). More importantly, these studies were conducted in adult subjects with an already established HPO axis. Relevant studies among adolescent patients with cancer are scarce (78, 79). A prospective study found GnRH-a administration during chemotherapy protected ovarian function and preserved fertility in adolescent patients (78). Another retrospective study drew a similar conclusion, with normal ovarian function maintained at the clinical, laboratory, and ultrasonic levels in 27/36 patients after GnRH-a co-administration (79). Overall, well-designed, large, prospective, randomized, controlled trials are essential to determine the protective effect of GnRH-a in children and adolescent patients (80).

Ovarian tissue cryopreservation

Ovarian tissue cryopreservation (OTC) is perhaps the sole option for fertility preservation in prepubertal children and post-pubertal adolescents who cannot delay the start of chemotherapy (11, 17, 32, 53, 81). Roughly 50% of the cortex from one ovary is surgically removed, dissected, and cryopreserved for future use (82, 83). The ovarian tissue is cryopreserved by either slow freezing (84–86) or vitrification (87). When a patient intends to restore ovarian function and/or fertility, the cryopreserved tissue can be thawed and replaced (Figure 1B).

In 1994, Gosden et al. successfully restored ovarian function to several castrated sheep using frozen-thawed ovarian slices (88). Symbolically, this has resulted in several lambs and maintained long-term ovarian function for up to 2 years (89). After that, studies of cryopreserved human ovarian tissue reported normal follicular morphology after thawing (90), follicular survival (91), and growth of follicles to antral stages (92) when replaced to immunodeficient mice. Thereafter, follicular growth (93, 94), ovarian endocrine function restoration (94), and *in vitro* embryo formation (95) were reported after being transplanted to humans. The first live birth after autologous ovarian tissue transplantation (OTT) using cryopreserved ovarian tissue in humans was documented in 2004 (96) and the second in 2005 (97). Since the milestone event, ovarian tissue cryopreservation and transplantation is gaining increasing attention in the field of fertility preservation. The cumulation of success has recently made it an accepted technique for fertility preservation (53).

Multiple transplantation strategies have been developed. The frozen ovarian tissue can be replaced either orthotopically (17) and/or heterotopically (98). Orthotopic sites include the remaining ovary (96) and peritoneal pockets created on the broad ligament (99). The orthotopic graft provides the possibility of spontaneous pregnancy because of the proximity to the fallopian tube (98, 100–103). Notably, the first live birth was conceived spontaneously

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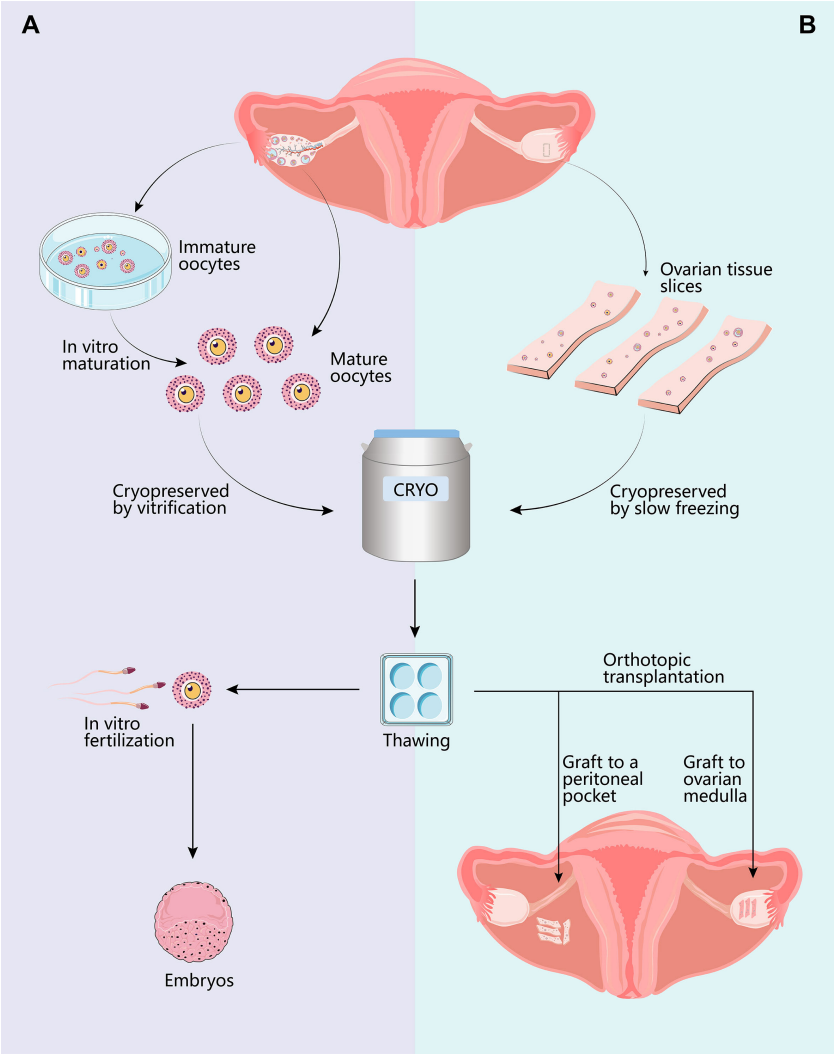

FIGURE 1
Options for female fertility preservation. For pubertal patients, mature oocyte cryopreservation is the optimal strategy. Controlled ovarian hyperstimulation and oocyte retrieval can be completed within two weeks if the treatments can be delayed. Another method requiring less time for ovarian stimulation is cryopreservation of *in vitro* matured immature cumulus-oocyte-complex (COCs). Additionally, immature COCs can be obtained while harvesting ovarian tissue for cryopreservation. Thawed oocytes are utilized for *in vitro* fertilization with intracellular sperm injection **(A)**, resulting in live birth rates per transfer varying between 39% and 52%. If the patient is prepubertal or requires immediate treatments, ovarian tissue cryopreservation remains the only option. The ovarian cortex is surgically removed, dissected, and cryopreserved. While vitrification is ideal for the cryopreservation of oocytes, slow-freezing is currently preferred for the preservation of ovarian tissue. Thawed ovarian slices may be transplanted either orthotopically or heterotopically **(B)**. Transplantation to orthotopic sites (broad ligament and ovarian medulla) provides the chance for spontaneous conception, whereas transplanting to heterotopic sites necessitates assisted reproductive techniques. The overall live birth rates after OTT range from 18.2% to 43.3%.

without either ovarian stimulation or *in vitro* fertilization (IVF) (96). In some studies, more pregnancies and live births were obtained naturally (99, 101, 102, 104). Many women have been reported to conceive and deliver more than once (104), with 3 cases delivering three times (105, 106) and one case conceiving four times (107). Heterotopic sites include subcutaneous areas in the forearm (98), abdomen wall (95), chest wall (100), breast (108), rectus muscle (108), and subperitoneal tissue (109), where a favorable environment for follicular development such as optimal temperature, paracrine factors, and blood supply may not be provided (100, 108). Thus, the procedure is adopted less frequently (110). Heterotopic autografting eliminates the possibility of conceiving naturally but not with the use of assisted reproductive techniques (ART). Clinical pregnancy (110) and live birth (111) following this procedure have been reported recently, partly removing its controversies. Meanwhile, the procedure also offers several potential advantages (100, 112), including, (1) less invasive surgery; (2) easier follicular monitoring and oocyte retrieval for IVF; (3) easier monitoring for cancer recurrence and removal of the graft, if necessary; and (4) more cost-effective options in case of repetitive transplantations. For some females who wish to restore ovarian function but do not desire pregnancy (98, 99), these advantages probably make heterotopic autograft a potentially preferred option.

Survival of grafted tissue and ovarian follicles depends on several factors, including the timing and location of transplantation, surgical techniques, and most importantly, the levels of revascularization soon after the procedure (113). Studies suggest that it takes 48 hours to revascularize after OTT in rodents (114, 115) but it may take up to 5 days in humans (115). In addition, research shows most follicles die before complete revascularization, with more than 70% of primordial follicles failing to survive the procedure in both humans (116) and sheep (89). There are challenges to further improving the survival of the graft and clinical outcomes (54, 113). On the other hand, cryopreserved ovarian tissue can be transplanted repeatedly in case of replantation failure (98). In a review including 318 women and 369 OTTs worldwide, Gellert et al. found that the average amount of transplanted tissue at the first OTT accounted for 46%, with 37% and 38% of the total amount of cryopreserved tissue being transplanted for the second and the third time, respectively (102). It seems a feasible strategy to extend the duration of ovarian function by repeating grafting procedures.

One of the leading concerns over the autograft of cryopreserved ovarian tissue is the risk of reintroducing malignant cells among malignancy survivors (100), which is considered high in hematological malignancies like leukemia and Burkitt lymphoma, and moderate in the case of Ewing sarcoma, advanced breast cancer, colon cancer, cervical adenocarcinoma (54, 112). In a recent systematic review, metastases were repeatedly detected in ovarian tissue obtained from patients with leukemia, but it was less common in other malignancies (117). Several methods have been applied to detect possible malignancy contamination before transplantation, such as histology (118), immunohistochemistry (119), and polymerase chain reaction (if specific markers are available) (117, 120). It has been proposed that ovarian tissue might be first xenografted to immunodeficient mice to assess the risk before grafted to humans (121). The recurrence rate after ovarian tissue graft in several large cohorts ranges between 3.9% and 7.0% (98, 99, 102), with a study comparing the relapse rate with those who did not accept transplantation and demonstrating similar recurrence rate (7%, 3/41 vs. 7%, 48/691) (99). None of these malignancy relapses was deemed related to OTT but dependent on the primary disease (98, 102), which has been endorsed by multiple studies (98, 102, 103, 107, 111, 122–126). Nonetheless, further studies are warranted to determine the safety of autograft of ovarian tissue among malignancy survivors (100, 102, 119, 121).

Males

Sperm cryopreservation

Sperm cryopreservation with masturbation is the easiest and most reliable method for fertility preservation for pubertal boys (11, 20, 33, 127, 128). Penile vibro-stimulation, as a noninvasive method, can be an alternative when having difficulties with masturbation (20). However, considering the invasiveness, electro-ejaculation and testicular sperm extraction (TESE) should be conducted only after weighing the benefits and harms (20, 129).

Cryopreservation of immature testicular tissue

Cryopreservation of immature testicular tissue (ITT) is the only fertility preservation option for prepubertal boys as spermatogenesis is absent (11). Small pieces of immature testicular tissue are surgically removed for cryopreservation. Yet still experimental, it is stressed that the procedure is provided exclusively for research purposes under ethical approval or novel technologies governance (20, 33, 76, 130). According to a survey, at least 1033 prepubertal boys aged between 3 months and 18 years have received the procedure (131). Multiple surveys reveal parents are willing to embrace the experimental technique (132–135), in hope that future advances in reproductive techniques will allow fertility restoration by the time their children have grown up (136). To date, however, comprehensive progress is still needed to make testicular tissue cryopreservation clinically applicable.

Testicular stem cells (TSC) can be stored in immature testicular tissue or a cell suspension. Detailed procedures of both strategies, including sample preparation, storage containers, cryoprotection, and cooling and warming process, have been elaborated elsewhere (137). Different cryopreservation strategies, including slow freezing and vitrification, have been attempted in human and animal models, leading to conflicting results (138–140). But slow freezing remains the most popular option for testicular tissue cryopreservation (130, 131), with both controlled (141–143) and uncontrolled (138) slow-freezing protocols under use.

The overall process of immature testicular tissue cryopreservation and fertility restoration procedures have been vividly described in a recent review (144). Potential methods for fertility restoration include autologous graft of immature testicular tissue (145), injection of testicular stem cells into the testis (146, 147), and *in vitro* maturation of TSCs (148, 149). The main advantage of ITT graft is the preservation of TSCs within their original niche (130). The maintenance of cell interaction and paracrine are preferable for tissue maturation, stem cell self-renewal, and differentiation (50, 150, 151). However, several male pediatric cancers, including testicular cancer, leukemia, and lymphoma, are prone to metastasize to the testes (152), significantly increasing the risks of malignancy relapse after autograft (33, 142). *In vitro* maturation of TSCs and reinjection of a TSC suspension free of malignant cells into testes, by contrast, can avoid the risks of cancer reoccurrence (50). But the original supporting conditions for *in vivo* spermatogenesis are absent.

Clinical outcomes after fertility preservation

Oocyte cryopreservation

For female patients, embryo preservation, if available, is the best method for female fertility preservation. However, lack of a permanent partner and ethical concerns to use of donor sperm make oocyte cryopreservation adopted far more frequently in post-pubertal adolescent patients (17). Poorer outcomes are seen compared to embryo cryopreservation due to oocyte degeneration

after thawing (53), which is even greater when it comes to immature oocyte cryopreservation (153–155).

The clinical outcomes using cryopreserved mature oocytes have been steadily improving as freezing/thawing techniques evolve and ICSI is used for fertilization (63). Some randomized control trials compared the clinical outcomes between vitrified oocytes and fresh oocytes, which confirmed the non-inferiority of vitrified oocytes to fresh oocytes in terms of fertilization rate, embryo development, implantation rate, clinical pregnancy rate, and live birth rate (62, 156), with similar conclusions drawn in other publications (61, 66–69, 157). The fertilization rates of oocytes with ICSI after thawing based on a large sample size ranged between 70.0% and 81.6% (61, 67, 158). The implantation rates fluctuated around 40% per embryo transferred (62, 67, 68). Notably, some studies found that the implantation rate using autologous vitrified oocytes was significantly lower than that of donor oocytes (63, 157). Current data suggests the clinical pregnancy rates per transfer can be as high as 50.7% to 62.6% (61, 63, 157, 159) whereas live birth rates per transfer range between 39% and 52% (61, 67, 157). A study found poor success rates among cancer patients than those who pursue elective oocyte preservation, but no statistically significant differences were observed after correction for age and controlled ovarian stimulation protocols (159). Current data collectively suggest that oocyte cryopreservation is an effective method for female fertility preservation. However, the efficiency of frozen/thawed oocytes remains unknown, which is vital for appropriate consultation regarding the number of oocytes to freeze to obtain at least a live birth in the future (61). Preliminary investigations revealed the overall percentage of warmed mature oocytes resulting in a live birth ranged between 4.2% and 10.8% (9.3 to 23.8 vitrified/thawed oocytes can lead to a live birth) (61, 63, 66). Besides, most previous studies were based on adult women aged >30 years, concluding that advanced age was negatively correlated with reproductive outcomes (61, 160, 161) and warranting more studies to counsel adolescent patients on the ideal number of oocytes needed to achieve a live birth (162).

While evidence indicates that advanced paternal age is less associated with increased rates of human embryonic aneuploidy (163, 164), it is well known that maternal age is highly correlated with oocyte/embryo aneuploidy (165) and it is one of the strongest predictors of IVF success (166). Interestingly, a recent study revealed that aneuploidy is also common among very young women (167). Gruhn et al. investigated the oocyte aneuploidy rates in women aged 9 to 43 years and found that oocytes aneuploidy rates from young women aged under 20 were significantly higher than those from women in their 20s and early 30s, which exhibited a U-shape curve. In comparison to women in their 20s to early 30s, younger women are also reported to experience higher rates of embryonic aneuploidy (165) and miscarriage (168), which deserves attention when providing counselling to post-pubertal adolescent patients on the clinical outcomes of oocyte cryopreservation.

Oocyte cryopreservation is an effective method for female fertility preservation. However, the relationship between long-term freezing and clinical efficacy, or offspring safety requires ongoing study (169). A multicenter study assessed the outcomes of oocytes cryopreserved for up to 48 months, no apparent differences in post-thawing oocyte survival, fertilization, cleavage, implantation, and live birth were observed when compared with those preserved for shorter periods (170). A more recent study reported a woman whose oocyte was frozen for 14 years and resulted in a healthy baby after fertilization with ICSI (171). On the other hand, congenital malformations were reported at rates ranging from 0.005% to 5.6% in several large cohorts (172, 173), which is close to the incidence in the USA national birth record in 2019 (3%) (169). Nonetheless, long-term follow-up of these children based on large cohorts is still needed.

Ovarian tissue cryopreservation

Currently, ovarian tissue cryopreservation is already considered an accepted technique for female fertility preservation given its success in restoring ovarian function and fertility (53). Recent studies demonstrated that reimplantation of ovarian tissue in the pelvic cavity resulted in the restoration of ovarian function in 85% to 95% of adult recipients (101, 103, 113, 174), as evidenced by the return of menstruation (98, 113, 175) or pregnancy (102, 113). Researchers also examined the serum hormone profiles before and after ovarian tissue transplantations, demonstrating a gradual decline in both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels and return to premenopausal levels 4 to 5 months after transplantation, which was accompanied by the resumption of menstrual cycles and the disappearance of menopausal symptoms (45, 99, 176, 177). However, restoration of ovarian endocrine function may not be reflected by AMH, which was almost undetectable in most cases (176, 178, 179), indicating a limited follicular population in the graft. According to Diaz-Garcia et al., the mean intervals between ovarian tissue transplantation and ovarian function resumption was 94.3 days (103), with most reported cases ranging between 3 and 6.5 months (98, 99, 104, 108, 123, 124, 176, 178, 180–182). The time frame of ovarian function resumption is consistent with that of folliculogenesis (183).

Duration of ovarian function after grafting can depend on the quantity of primordial follicles at the time of transplant and proportion that survive the grafting process (99). The mean duration is approximately 4 to 5 years in humans (174). However, in a study including 41 young women (aged 32.9 on average at the time of OTT), more than half of the transplantations resulted in a functional life span between 1 and 4 years, with some cases lasting for more than 10 years while several cases lasting less than one year (99). The longest duration of restored ovarian function recorded to date is 13.5 years by repeating the transplantation procedure (98). The heterogeneity indicates the necessity of improving and standardizing procedures for ovarian tissue cryopreservation and transplantation.

Since the first live birth report after ovarian tissue autografting in 2004 (96) and the second in 2005 (97), the number of pregnancies and live birth have continued to climb steadily, showing an exponential trend (82). Live births after autografting

of ovarian tissue cryopreserved before (184, 185) and after (186, 187) menarche have been reported recently. The number of live births after ovarian tissue transplantation was estimated to exceed 200 in 2020 (188). However, the total number of transplantations worldwide (the denominator) is unknown, leading to the unavailability of accurate pregnancy rates and live birth rates. In an early study based on five centers worldwide including 111 patients, the pregnancy rate and live birth rate were 29% and 21%, respectively (105). These figures were subsequently confirmed by several case series and pooled analyses with larger sample sizes (98, 99, 101–104, 113, 182), yielding a clinical pregnancy rate between 27.3% and 65.6%, and a live birth rate between 18.2% and 43.3%, respectively. Nonetheless, these results have been confounded by both patient factors (i.e. age at OTC/OTT, exposure to gonadotoxic therapy, the number and size of ovarian slices replaced, and residual ovarian function, *etc.*) and technical factors (i.e. surgical techniques, application of proangiogenic agents, the assistance of artificial reproductive techniques, *etc.*) (100).

Currently, most studies focus on adult subjects with little attention to ovarian tissue cryopreservation and transplantation from tissue taken from prepubertal children and post-pubertal adolescents. There are several large cohorts of young girls reporting ovarian tissue cryopreservation, but the return-to-use rates are extremely low (45, 189, 190), leaving limited data to evaluate the endocrinological and reproductive function after ovarian tissue replantation in this population. Table 2 includes some of the current reports on ovarian tissue transplantation that were cryopreserved at the age of ≤ 20 years. In 2012, there were two cases of ovarian tissue transplantation in pre-pubertal girls to induce puberty (191, 192), resulting in gonadotropins decline and estradiol secretion. Although ovarian activity ceased about 2 years after the grafting, both patients established normal menstrual cycles and secondary sex characteristics shortly after the grafting, demonstrating proof of concept in inducing puberty. As the amount of tissue required for pregnancy and parenthood is unknown in any individual, concerns regarding use of OTT for pubertal induction remain (198, 199).

Another subject of study is the relationship between follicular density/quantity and the longevity of restored ovarian function. Several studies observed an association between younger age at the time of ovarian tissue cryopreservation and preferable clinical outcomes including the longevity of graft survival (200) and live birth rates (102, 103, 113, 196), though challenged by another study (99). The association may be, at least partly, explained by the greater number of follicles residing in the tissue upon harvest in the younger population. In fact, the primordial follicle pool in cryopreserved ovarian tissue retrieved from prepuberty adolescents is significantly larger than those from older patients (189, 201). However, in the report by Ernst et al. (191) and Poirot et al. (192), the ovarian tissue was cryopreserved at the age of 9 and 10 years, respectively. After the graft, however, the endocrinological function was maintained for merely 19 months and 2 years, respectively, much shorter than the average duration reported in adult subjects. These discrepancies mean much optimization is still needed to maximize the clinical outcomes.

Notably, some patients may undergo chemotherapy before ovarian tissue cryopreservation. The latest studies have demonstrated similar resumption in ovarian function and pregnancy rates per woman in patients who received low gonadotoxic risk chemotherapy compared to those who were chemo-naïve (98, 104, 182). Importantly, ovarian harvest in those who have achieved complete remission following chemotherapy may reduce the chance of malignant contamination among patients with leukemia (117–119). However, chemotherapy containing alkylating agents does adversely impact the clinical outcomes including both the pregnancy rate and live birth rate (98).

Sperm and testicular tissue cryopreservation

Sperm cryopreservation is the most successful method for male fertility preservation. While some studies suggested reduced sperm viability and motility after thawing (202, 203), cryopreserved sperm from patients with a previous malignancy has comparable potential to obtain a clinical pregnancy as ART evolves, especially with the use of ICSI for fertilization (204, 205).

The documented clinical pregnancy rates using thawed sperm collected before cancer therapy ranges from 18% to 57% (204, 206–210). Meanwhile, a higher success rate is observed in ICSI programs, followed by IVF, with intrauterine insemination (IUI) being the least successful (206–208, 210). According to an early study, it took a median of 3 cycles to get pregnant in ICSI, whereas 8 cycles were required in IVF (211). Similar pregnancy rates have been observed compared with non-cancer control or fresh sperm (205, 208). A large cohort involving 272 males with cancers reported a live birth rate of 62.1% per patient, comparable to the non-cancer infertile population (205). Specifically, the outcomes of couples using testicular sperm do not differ between fresh and frozen-thawed sperm among patients with Klinefelter syndrome (212, 213), obstructive azoospermia (214), and non-obstructive azoospermia (215, 216). But the cumulative live birth rate was lower than that of ejaculated sperm (216, 217).

Sperm can be stored for decades under ultra-low temperatures. The longest duration of sperm cryopreservation to date is 28 years, which successfully resulted in a healthy live birth with IUI (218). Unfortunately, it remains unknown whether the freezing-thawing process poses an adverse impact on the long-term development of children born with cryopreserved sperm.

For those patients who had their testicular tissue cryopreserved, the fertility restoration strategy includes autologous grafting of immature testicular tissue (145), injection of TSCs into the testis (146, 147) and *in vitro* maturation of TSCs (148, 149) and these options have been extensively discussed in a recent review (130). Due to its experimental nature, clinical outcomes on testicular tissue transplantation in human subjects are still unavailable regardless of great achievements obtained using animal models (20, 33, 50).

Transplantation of immature testicular tissue appears to be one of the most promising methods for male fertility preservation. Since the live birth of mice and rabbits after fresh and cryopreserved

TABLE 2 Current information about ovarian tissue cryopreservation and transplantation in prepubertal children and post-pubertal adolescents.

Reference	Age at OTC	Age at OTT	Hormonal restoration	Interval between graft and restoration	Duration of ovarian function	Pregnancy	Live birth	Notes for results
Ernst et al. 2013 (191)	9	13.5	YES	4 Mon	19 Mon	NA	NA	NA
Matthews et al. 2018 (185)	9	23	NA	NA	NA	YES	YES	IVF
Poirot et al. 2012 (192)	10	13	YES	2 Mon	2 years	NA	NA	NA
Demeestere et al. 2015 (184)	14	24	YES	4.5 Mon	NA	YES	YES	NC
Meirow et al. 2016 (107)	14	21	NO	–	–	NO	NO	Graft failure
	19	27	YES	NA	4 Mon	NO	NO	IVF failure
	19	31	YES	NA	NA	YES	NO	Ongoing pregnancy
	19	37	YES	NA	NA	NO	NO	IVF failure
Donnez et al. 2011 (193)	17	24	YES	3.5 Mon	NA	YES	YES	NC
	20	23	YES	4 Mon	NA	YES	YES	NC
	20	NA	YES	3.5 Mon	>8 Mon	YES	YES	NC
Póvoa et al. 2016 (194)	18	28	YES	1 week	>6 Mon	NO	NO	Embryo cryopreserved
Donnez et al. 2012 (186)	18	28	YES	24 weeks	NA	YES	YES	IVF
Rosendahl et al. 2011 (122)	NA	19	YES	NA	>18 Mon	NA	NA	Embryo transfer
Revel et al. 2011 (187)	19	23	YES	NA	>9 Mon	YES	YES	IVF
Schmidt et al. 2011 (176)	19	NA	YES	16 weeks	>18 Mon	NO	NO	IVF failure
Roux et al. 2010 (195)	20	23	YES	9 weeks	NA	YES	YES	NC
Van der Ven et al. 2016 (196)	20	27	YES	NA	>1 year	YES	YES	NC
	20	29	YES	NA	>1 year	YES	NO	NC, Tubal pregnancy
Callejo et al. 2013 (197)	20	30	YES	4.5 Mon	NA	YES	YES	IVF

OTC, ovarian tissue cryopreservation; OTT ovarian tissue transplantation; NC, natural conception; IVF, *in vitro* fertilization

immature testicular tissue transplantation in 2002 (219). Achievements have been made on various animal models, with promise toward clinical use. In summary, functional spermatogenesis after testicular tissue graft has been made possible in mice (220), ferret (221), sheep (222), pigs (223), collared peccary (149), bison (224), buffalo (225), Coturnix japonica (226), and in non-human primates including marmoset (227), cynomolgus monkey (228), and rhesus macaques (145, 229). In some species, offspring using graft-derived sperm with ICSI have been reported (145, 223, 226, 228), proving its potential in fertility preservation and restoration in prepubertal males. The anatomy and physiology of the testis in non-human primates resemble humans the most and make them perfect preclinical models for ITT transplantation research (130). Most recently, fresh and cryopreserved testicular tissues from prepubertal rhesus macaques were autologously transplanted under the back skin and scrotal skin after castration. Surprisingly, all grafts survived, grew, and restored testosterone reproduction as well as endogenous spermatogenesis. A healthy female baby was produced with graft-originated sperm (145). This study marks the biggest milestone for testicular tissue cryopreservation and auto-transplantation toward clinical translation.

Reinjection of TSCs was first introduced in 1994 (146, 147). TSCs isolated from immature mice were injected into the testes of infertile hosts and successfully colonized the seminiferous tubules. The host mice restored natural spermatogenesis and produced offspring using sperm from donor tissue. From that onwards, the method has been proven successful in multiple animals. Offspring

were obtained by either natural mating or assisted reproductive techniques after fertility recovery in mice (230, 231), rats (232, 233), goats (234), sheep (235), chicken (236), and zebrafish (237). However, the overall success using primate models is limited. *In vivo* spermatogenesis was recovered (229, 238, 239), and in some cases, embryo formation was documented (238), but no offspring have been reported yet.

In vitro maturation of TSCs was also established on mice models which successfully resulted in offspring using round spermatids with ICSI (230). Researches focusing on rats (240), pigs (241), calf (242), and buffalo (243) has successfully induced post-miotic cells (haploid germ cells), and some studies progressed to the formation of preimplantation blastocysts (244). But healthy offspring was reported in mice exclusively (130). IVM of TSCs in non-human primates (245, 246) and humans (247–249) has led to similar results. Only post-meiotic cells were documented. Overall, this technique is still in its infancy.

Transgender population

Transgender individuals represent a special population who recognize internal gender as different from biological gender. The latest statistics estimated that there are 150,000 young and 1.4 million adult transgender women (transwomen, MtF) or transgender men (transmen, FtM) in the United States (250). In addition, there is a trend towards presentation at younger ages (251). To alleviate gender dysphoria, many of them choose gender-affirming therapy including gender-affirming hormone therapy (GAHT) and gender-affirming surgery (GAS) (43), rendering temporary subfertility or permanent sterility (252). Accordingly, gender-affirming therapy, both hormonal and surgical, is one of the indications for fertility preservation (43, 253).

A variety of studies suggest transgender individuals have a strong desire for parenthood. 62% to 82% of transgender individuals want to have children, biological or adopted (254–256). But the desire to have children declines throughout the GAHT process (257). Meanwhile, nearly half of the transgender adolescents noted that their desire to have their biological children may change when they grow up (258) whereas a proportion of them regretted not undergoing fertility preservation (259, 260). A recent survey revealed that almost all (94.6%, 387/409) transgender respondents agreed that fertility preservation should be offered to all transgender individuals (261).

Several scientific societies have issued guidelines navigating health care to the transgender population, recommending that all transgender individuals should receive a consultation about potential fertility risks of gender-affirming treatments and preservation options before transition (43, 253, 262). However, no guideline specifies the optimal time to initiate discussion and counseling, leading to some situations where patients have their first discussion about fertility preservation after the initiation of gender-affirming therapy (263, 264). Inadequate and belated information provision puts patients in a dilemma between fertility preservation and discontinuation/delay of gender-affirming therapy. Considering most transgender persons are reluctant to postpone or suspend gender-affirming therapy (254, 261), it appears even more important to start consultation as earlier as possible.

Fertility preservation for transmen

Oocyte cryopreservation, embryo preservation, and ovarian tissue cryopreservation are established methods for fertility preservation for transmen. While ovarian stimulation and the accompanying unpleasant experience of estradiol elevation, vaginal examination and oocyte retrieval are unavoidable for oocyte cryopreservation (265, 266), ovarian tissue can be obtained at the time of gender-affirming surgery. For transmen who have not started GAHT, ovarian stimulation protocols are the same as those used for infertility (251). GnRH antagonist protocol can be considered for its efficacy in oocyte yield (32), and the addition of letrozole can reduce estradiol levels and related symptoms (267).

Some transmen may have already started testosterone treatment before ovarian stimulation. It was previously deemed that testosterone induced polycystic ovary syndrome (PCOS) (268). But recent studies demonstrated testosterone exposure for more than a year did not disturb ovarian follicle distribution (269, 270). Some studies suggest temporary discontinuation of hormonal therapy before ovarian stimulation (251, 265). While some small studies demonstrated no differences in oocyte yield between transmen with continuous hormonal therapy and ciswomen (271–273).

In vitro maturation of immature oocytes from oophorectomy can be another source of oocytes (274). However, a recent study demonstrated the low feasibility of this strategy for fertility preservation (275). Almost 2,000 cumulus-oocyte-complex (COCs) were collected at the time of oophorectomy and merely 23.8% of them matured after *in vitro* culture. Of the 151 out of 208 mature oocytes that survived vitrification/thawing, 139 oocytes were fertilized with ICSI, leading to 48 normal fertilizations (34.5%) and 4 transferable blastocysts. Collectively, given the poor maturation rate (28% to 36%) and utilization efficacy after IVM (59, 276), as well as lower pregnancy rates and higher pregnancy loss rates (277–279), IVM should not be used as the only method for fertility preservation in transmen (280).

For prepuberty transmen and those who are unwilling to accept ovarian stimulation, ovarian tissue cryopreservation is the sole option for fertility preservation (253). Ovarian tissue can be obtained after oophorectomy without testosterone discontinuation (280). Auto-transplantation of cryopreserved ovarian tissue has resulted in more than 200 live birth (188). But there is no report of ovarian tissue transplantation in transmen.

Fertility preservation for transwomen

Sperm cryopreservation is a reliable option for fertility preservation among transwomen (253). Sperm may be obtained by either masturbation, assisted ejaculation, or TESE (33, 129). Cryopreserved sperm could be used for IUI, alternatively, IVF/ICSI using oocytes from a donor or cisgender female partner (251).

Cumulative live birth rate using frozen/thawed sperm before anticancer treatment can be as high as 62.1% with ART (205), but these results may not apply to the transgender population because gender-affirming therapy (280–282) and some behavioral factors (283, 284) pose reversible or irreversible threats on semen quality.

For prepubertal transgender girls, testicular tissue cryo preservation remains the only option for fertility preservation (251). Some transgender girls may have received puberty suppression and estrogen supplementation at different pubertal stages (43), leading to decreased testosterone levels. The deficiency of intratesticular testosterone results in severe spermatogenesis dysfunction (280). The effect of testosterone suppression on spermatogenesis in adults have been extensively investigated in cisgender male contraceptive research and is reversible after cessation of suppression therapy (285). But the effect on pubertal transgender girls remains partly unanswered. de Nie et al. investigated the histology of testes using orchiectomy samples under testosterone suppression and/or estrogen exposure (286). They found only immature germ cells (spermatocytes and spermatogonia) present in the seminiferous tubules when medical intervention started at Tanner stage 2-3, with additional mature sperm observed in 57% of subjects who initiated medical treatment at Tanner stage 4 or later. These findings indicate the potential of testicular tissue cryopreservation for fertility after the initiation of puberty suppression and estrogen therapy. However, testicular tissue cryopreservation is currently experimental (131). Future use depends on advances in IVM of testicular stem cells since the *in vivo* microenvironment for spermatogenesis is unobtainable after orchiectomy.

Fertility preservation program

Current challenges

Several scientific societies have issued clinical practice guidelines for fertility preservation in pediatric and adolescent cancer populations (11, 17, 32, 50, 76), but surveys indicate limited knowledge of guidelines and poor compliance with recommendations by medical professionals (12, 13, 287, 288). According to a survey in the United States, only 46% and 12% of oncologists routinely refer male and female pubertal patients to fertility preservation services before cancer treatment, respectively (289). Similar research among adolescent and young adult cancer survivors reveals that 80% and 68% of male patients can recall being offered information about potential fertility impairment and referral to fertility preservation service, but the figures for female patients are only 48% and 14%, respectively (290). Evidence suggests that most patients and their parents are dissatisfied with the content of information that healthcare professionals provided concerning fertility risk and available options to preserve it (17). Younger patients and their parents are concerned about fertility issues, but they find it difficult to extend discussions with their physicians (291–293). A major barrier hindering preferable fertility preservation practice is the lack of a structured and coordinated fertility preservation program (12, 13). Meanwhile, some well-organized fertility preservation programs have been proven very successful (294–297).

Education for health professionals

Clinicians' knowledge and attitudes toward fertility preservation significantly influences fertility preservation practice (12, 13, 288, 298). Physicians may be restrained by their limited understanding of the gonadotoxic nature of chemotherapy or radiotherapy, potential risks regarding future family planning patients may face, fertility preservation possibilities, and the highly time-sensitive nature of this intervention (12, 288, 299). While some physicians have realized the sensitivity and importance of fertility issues, oncologists tend to provide treatments that maximize the chance of survival and regard fertility issues as a non-priority (12, 76). However, based on the increasing proportion of children and adolescents who survive malignancies (1–3), it is crucial to focus on patients' quality of life (294, 300) and incorporate fertility preservation into cancer care (76, 301). Accomplishing the objectives should start with educating healthcare professionals (12, 76, 295), possibly including pediatric oncologists, radiation oncologists, gynecologists, urologists, hematologists, surgeons, nurses (11, 32, 127, 302). The clinical team is supposed to have sound knowledge about infertility risk assessment and fertility preservation consultation (11, 12, 17, 32, 76) as fertility risk assessment of specific agents or therapy regimes has been extensively discussed in previous guidelines (25, 47–50). Some of the most common gonadotoxic agents are presented in Table 3. To better facilitate fertility preservation, it is suggested to be incorporated into general education of oncology (76, 294, 302). According to a recent study, a simple fertility training program can considerably increase oncologists' knowledge of infertility risk assessment and fertility preservation strategies (303).

Informed consent

Discussion about potential fertility risks and methods to preserve it must begin at the time of diagnosis (mostly at the time of cancer diagnosis) (11, 76). It may be necessary to assume that

TABLE 3 Estimated risk of chemotherapy for gonadal function (46).

High risk	Medium risk	Low risk
Cyclophosphamide	Cisplatin	Vincristine
Ifosfamide	Carboplatin	Methotrexate
Chlormethine	Doxorubicin	Dactinomycin
Busulfan		Bleomycin
Melphalan		Mercaptopurine
Procarbazine		Vinblastine
Chlorambucil		

every young patient diagnosed with a disease that requires gonadotoxic therapy is concerned with fertility issues (127). One approach to promote fertility preservation practice would be to make it the clinicians' (pediatricians and oncologists in particular) regular responsibility to evaluate potential fertility risks and initiate discussions about fertility preservation including options, benefits, risks, and costs with patients (53, 294). It is proposed that the clinical team assign a knowledgeable team member to offer a comprehensive and in-depth consultation, providing detailed information concerning potential risks of fertility loss with proposed treatment regimes, current options for fertility preservation, possible risks of delaying treatment, the overall prognosis of cancer, and location of local or regional fertility preservation service, and be ready to answer questions that patients are interested (294).

A formal and separate discussion about fertility issues is advocated to ensure patients' full comprehension of potential fertility risks and fertility preservation options (136, 294). Importantly, considering the sensitive nature of the age group and topics, patients should be offered a chance to speak freely without the presence of their parents (136, 294). In addition, institutions can design printed or online education materials for interested patients and the ordinary public to facilitate information provision (32). Notably, both formal conversation and supplementary materials should be organized in lay languages and avoid professional terms to ensure that recipients can comprehend the information (127, 294). The provision of relevant medical information should be documented in the patient's medical record and patients who decide to seek fertility preservation must provide written informed consent (32, 294).

Referral

The establishment of a standardized intra-institutional or inter-institutional referral pathway between the clinical team and fertility preservation team is strongly suggested (32, 53). In addition, a coordinator between both teams would play a crucial role in navigating the fertility preservation process (32, 127). This institutional arrangement has several advantages.

Firstly, it facilitates fertility preservation consultation (32, 294, 304). Concerns by oncologists about lack of efficacy can hinder referral of patients to fertility preservation services (12), thus, collaboration with fertility specialists allows the chance to eliminate their doubts and update them on the latest fertility preservation technologies (76). Furthermore, fertility preservation specialists can contribute to fertility education provided to oncologists as described above.

Secondly, it facilitates referral (32, 294). Another barrier hindering oncologists' raising fertility discussion is that they are not aware of local or regional fertility services (12, 302). A direct link between the clinical team and the fertility preservation team would greatly facilitate referral. For instance, a male patient who wishes to bank sperm can make first contact with the sperm bank (294, 296, 304). This would not only save patients time and costs upon the life-changing event (cancer) but also reduce their psychological stress.

Thirdly, it enables optimal decision-making (76). The fertility team is proficient in fertility preservation techniques. But they must cooperate with the clinical team to decide whether it is medically feasible to perform fertility preservation procedures and determine the optimal timing and strategy (53, 76, 304).

Finally, it reduces repetitive work and improve efficiency. As described above, a fertility specialist can engage in both patient counseling and proposing a feasible fertility preservation plan with the clinical team, saving their time and energy. A fertility specialist can focus on other affairs concerning fertility preservation, such as logistics of the procedure, cryopreservation of relevant materials, and future use. Considering the highly time-sensitive nature of fertility preservation, any improvement in efficiency would benefit patients significantly.

Effectiveness and efficiency

Based on previous literature, there are two identified key factors in establishing an effective and efficient fertility preservation program, namely sensitive faculties (294, 297, 304, 305) and a rapid referral pathway (294, 295, 304). Those patients who require fertility preservation do not generally visit a reproductive clinic. Instead, most of these patients are identified in a pediatric or an oncological clinic. The optimal strategy would be educating pediatricians, oncologists, and relevant nurses to raise their sensitivity to fertility issues (32). More importantly, they should be familiar with common gonadotoxic therapy so that they can assess fertility risks and decide whether to refer a patient or not and fertility preservation can be moved forward quickly. Moreover, a rapid referral approach would substantially increase patients' awareness and accessibility to fertility preservation services. Patients and their families tend to feel overwhelmed upon the diagnosis, sparing them limited time and energy to access information concerning fertility risks and ways to preserve it (294). As mentioned previously, even some medical professionals lack the necessary knowledge about the potential fertility risks of chemotherapy and radiotherapy and available local or regional fertility preservation services (12, 13, 302). It is reasonable to assume most patients and their families are ignorant of these risks and relevant services. A rapid referral pathway significantly reduces the time and costs patients need to access fertility preservation services. The role of the fertility preservation team is relatively dependent on referral since patients do not generally visit them. However, once engaged, they can work with the clinical team and propose an optimal fertility preservation scheme as soon as possible. Given the urgency of both cancer therapy and fertility preservation, any delay in the process would potentially harm patients' interests.

Future directions

Although different communities have made great achievements in fertility preservation, there are still some restrictions in clinical practice. For instance, unlike embryo or gamete cryopreservation, transplantation of cryopreserved ovarian tissue generally leads to variable outcomes between patients and institutions, warranting optimization and standardization of this technique. Currently, the greatest challenge is the massive follicle atresia after transplantation, with more than 70% of primordial follicles failing to survive (116). Future studies should focus on promoting follicular survival by enhancing revascularization and reducing ischemia-reperfusion injury soon after the transplantation (54). Potential strategies include transplanting ovarian tissue with biocompatible decellularized extracellular matrix scaffold (306), and the utilization of antioxidants, anti-apoptotic agents (307, 308), or proangiogenic factors (309, 310). In addition, artificial ovary (311) and complete *in vitro* development of follicles (312) are potential strategies for female fertility restoration. Recent advances in testicular tissue autograft from prepubertal rhesus macaques are encouraging (145), highlighting its promise in male fertility restoration but more similar studies are required to move it towards open clinical trials. Meanwhile, additional efforts are required to address the risks of malignancy reintroducing and investigate the long-term health of children born through fertility preservation programs. Finally, disciplines must collaborate to set up an effective and efficient fertility preservation program that enhances awareness among medical professionals and patients and removes the barriers to fertility preservation services.

Conclusions

In conclusion, fertility preservation is an increasingly important issue in pediatric and adolescent healthcare as most malignant diseases are curable with contemporary means. Mature gamete cryopreservation is the most reliable and successful strategy for fertility preservation when embryo freezing is not practicable. In addition, ovarian tissue cryopreservation is an effective option for female fertility preservation though there is room for improvement in its efficacy. Autograft of immature testicular tissue is currently the most promising method for prepubertal patients, but additional efforts are required to gather data on animal models and in clinical trials.

Author contributions

LC, ZD and XC: Substantial contribution to the conception and design of the work. LC: Participation in the acquisition of literature. LC and XC: Manuscript drafting and revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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National oncofertility registries around the globe: a pilot survey

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Purpose: Oncofertility is an emerging discipline which aims to preserve fertility of young cancer patients. As fertility preservation services have become increasingly available to cancer patients in many countries around the globe, it is crucial to establish a foundation of collaborative reporting to continuously monitor and assess oncofertility practices. This survey study investigates the current global landscape of official national oncofertility registries, a vital tool which allows for surveillance of the field.

Methods: An online pilot survey was conducted to give the opportunity to report official national oncofertility registries available in 2022. Survey questions covered the availability of official national registries for oncofertility as well as the official national registries for cancer and assisted reproductive technologies. Participation in the survey was voluntary, anonymous and for free.

Results: According to our online pilot survey, responses were collected from 20 countries including Argentina, Australia, Brazil, Canada, Chile, China, Egypt, Germany, Greece, India, Japan, Kenya, Philippines, Romania, South Africa, Thailand, Tunisia, UK, USA & Uruguay. Only 3 out of the 20 surveyed countries have well-established official national oncofertility registries; and include Australia, Germany & Japan. The Australian official national oncofertility registry is part of Australasian Oncofertility Registry that also includes New Zealand. The German official national oncofertility registry is part of FertiPROTEKT Network Registry for German speaking countries that also includes Austria & Switzerland. The Japanese official national oncofertility registry includes Japan only and called Japan Oncofertility Registry (JOFR). A supplementary internet search confirmed the aforementioned results. Therefore, the final list of countries around the globe that have official national oncofertility registries includes Australia, Austria, Germany, Japan, New Zealand, and Switzerland. Some other countries such as the USA and Denmark are on their way to establish official national registries for oncofertility care.

Conclusion: Although oncofertility services are expanding globally, very few countries have well-established official national oncofertility registries. By reviewing such a global landscape, we highlight the urgent need for having a well-established official national oncofertility registry in each country to monitor oncofertility services in a way that best serves patients.

KEYWORDS

oncofertility, registry, database, fertility preservation, cancer

1 Introduction

Advancements in medicine have led to an increase in the survival rates of pediatric and adolescent cancers over the past decades (1). While these patients are recovering and living longer, common cancer treatments such as alkylating chemotherapy and ionizing radiation are highly gonadotoxic, resulting in fertility loss and a low chance of genetic parenthood for many survivors. The risk of cancer therapy-induced gonadotoxicity and fertility loss depends on the type and dose of cancer therapy, type and stage of the disease, as well as the age of the patient and the status of reproductive functions at the time of treatment (2). Therefore, there is a growing need to develop novel techniques which allow for the preservation and protection of fertility for pediatric, adolescent, and young adult cancer survivors. First coined in 2006, "Oncofertility" is an interdisciplinary field that bridges oncology and reproductive medicine with the goal of preserving the reproductive future of cancer survivors (3). The increasing availability of new oncofertility services such as ovarian and testicular tissue cryopreservation, in addition to the traditional sperm, egg and embryo cryobanking has generated a need to monitor these services across institutions and clinics.

Registries crucially provide an unparalleled opportunity for the surveillance of medical practices at all levels- local, regional, national, multinational, and globally. When registries are thorough, and data is complete and accurate, the information contained has the potential to shed light on epidemiological trends, highlight areas for improvement, and inform public health stakeholders. In any field, well-established national registries create opportunities for the surveillance and comparison of treatments on a large scale, allowing for the evaluation of novel approaches, and the continued safety monitoring of traditional approaches across the country. Ultimately, the information contained within a well-established national registry has the potential to guide informed decision making for both patient and provider, improve patient experience and reduce the burden of disease (4, 5).

Some of the most well-established registries are in the field of oncology (5). Such registries hold information on cancer prevalence, subtypes and treatment efficacy which have guided standards of care (6). For example, a study on registry data reported a marked disparity in mortality from cancer between developed and developing countries, with 57% of new cases and 65% of deaths in 2012 occurring in developing countries, painting a global picture that would have otherwise been more difficult to obtain (7). CONCORD, an established global surveillance program for cancer survival trends across 71 countries collected data from over 37.5 million cases in a 15-year period, which was used to inform on the global status of cancer and guide healthcare policy, speaking to the clear benefit of medical registries (8–10).

Compared to cancer registries, the degree to which Assisted Reproductive Technologies (ART) registries are established is more varied. The European IVF monitoring consortium is one such registry used to track the success of multiple reproductive technologies performed at over 1000 institutions from 43 countries (11). Nearly annual reports on this registry have uncovered a trend towards marginal improvement in efficacy of these services, among other findings (12–15). The quantity of data collected in this registry increases the impact of its studies and further highlights the importance of ART registries.

While some ART registries may include specialized oncofertility services, there is a general lack of recording this data in many countries around the globe. The main purpose of this study is to investigate the global existence of national oncofertility registries in relation to national cancer and ART registries.

2 Methods

2.1 Data collection

In order to identify cancer, ART and oncofertility official national registries, we conducted an online pilot survey in 2022, asking participants to report the registries available in their countries by answering the survey questions as shown in Figure 1.

The survey study was designed and conducted by the Oncofertility Consortium team at Michigan State University (MSU), USA. The Institutional Review Board (IRB) at MSU determined that this survey study did not constitute research that involves human subjects; therefore, additional IRB review and approval was not required.

The survey was available online on MSU Qualtrics from January 2022 to September 2022. Furthermore, a link to the online survey https://msu.co1.qualtrics.com/jfe/form/SV_brTwsVJ3vVsLqdw was shared at the 14th Annual Conference of the Oncofertility Consortium, May 2-4, 2022, Pittsburgh, PA, and in the Oncofertility Consortium e-newsletters that may appeal to oncofertility care providers, such as those sent by related professional societies and academic departments.

Participation in this online survey was voluntary, anonymous and for free. Responses were confidential and no identifying information such as personal names, email addresses or IP addresses was collected. All data was stored in a password protected electronic format for scholarly purposes only.

2.2 Data analysis

Upon closing the survey period, we conducted a thorough internet search to authenticate each response and check whether each national registry officially exists. In the cases where survey participants from the same county reported different information about registries, all responses were reviewed to determine the accuracy of the data. Responses found to be inaccurate were discarded. After data cleaning, descriptive statistics were used to analyze the final dataset.

3 Results

Responses from 20 countries were collected via the online survey including Argentina, Australia, Brazil, Canada, Chile,

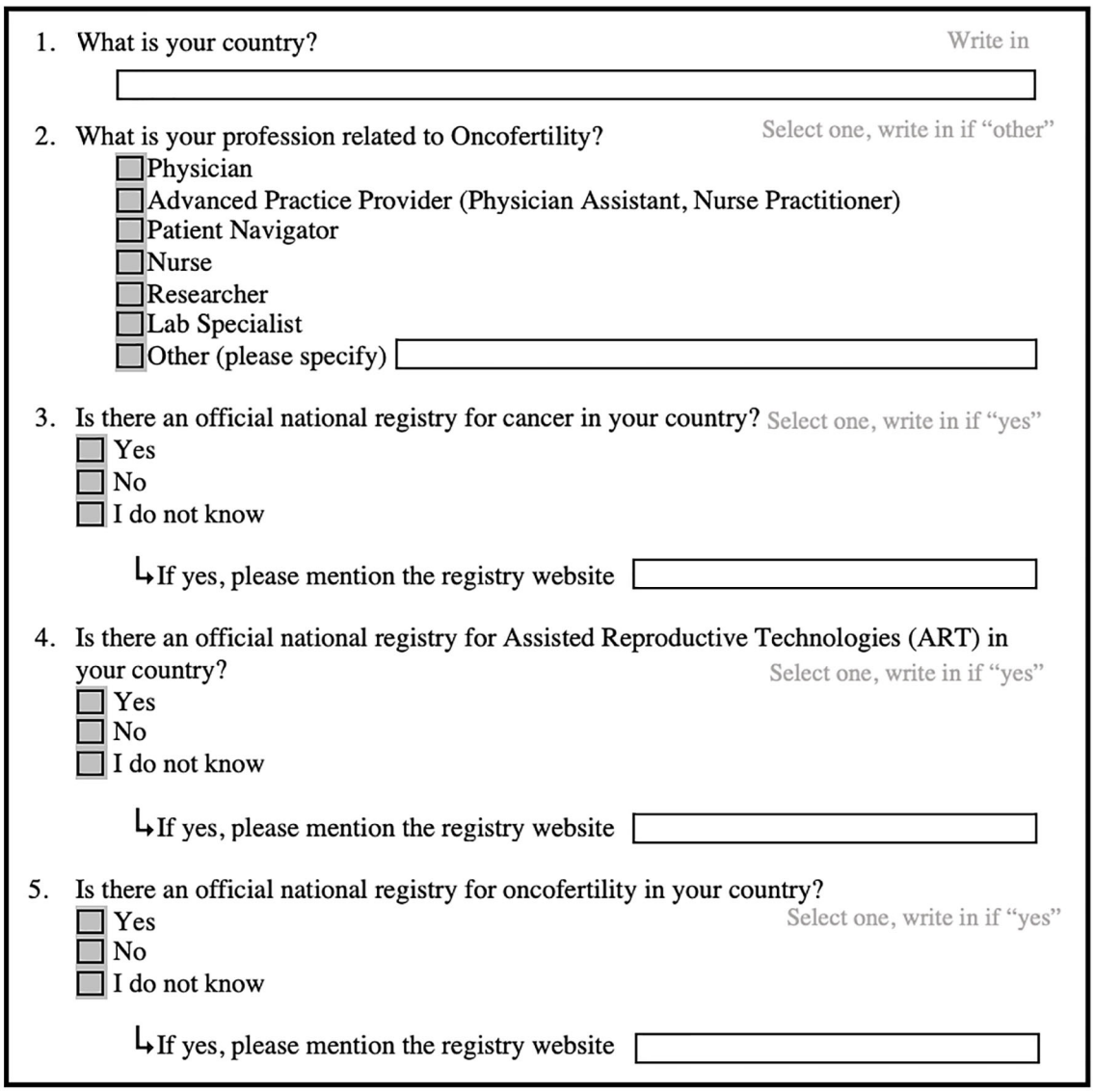

FIGURE 1
Survey Questions.

China, Egypt, Germany, Greece, India, Japan, Kenya, Philippines, Romania, South Africa, Thailand, Tunisia, UK, USA & Uruguay. Twelve countries (60%) have official national cancer registries including Argentina, Australia, Brazil, Canada, Germany, India, Japan, South Africa, Thailand, UK, USA & Uruguay. Thirteen countries (65%) have official national ART registries including Argentina, Australia, Brazil, Canada, Germany, Greece, Japan, Romania, South Africa, Thailand, UK, USA & Uruguay. Only 3 countries (15%) have official national oncofertility registries including Australia, Germany & Japan (Table 1).

4 Discussion

According to our online pilot survey, responses from 20 countries were collected and showed that only 3 countries (Australia, Germany & Japan) have well-established official national oncofertility registries. The Australian official national oncofertility registry is part of Australasian Oncofertility Registry (AOFR) that also includes New Zealand (http://www.futurefertility.com.au/registry/). The German official national oncofertility registry is part of FertiPROTEKT Network Registry for German speaking countries that also includes Austria & Switzerland (https://fertiprotekt.com/en/patients/). The Japanese official national oncofertility registry includes Japan only and called Japan Oncofertility Registry (JOFR) (http://www.j-sfp.org/about/registry.html).

To supplement this pilot survey, we conducted a separate internet search to identify further official national oncofertility registries around the globe. Keywords were searched on both PubMed and Google alongside the countries' names and "registries" (ex: "USA oncofertility registries"). Key terms included: oncofertility, cancer, oncology, assisted reproductive

TABLE 1 Results of the online pilot survey.

Countries involved in the online pilot survey	Countries with an official national cancer registry	Countries with an official national ART registry	Countries with an official national oncofertility registry
N = 20 (100%)	N = 12 (60%)	N = 13 (65%)	N = 3 (15%)
1. Argentina 2. Australia 3. Brazil 4. Canada 5. Chile 6. China 7. Egypt 8. Germany 9. Greece 10. India 11. Japan 12. Kenya 13. Philippines 14. Romania 15. South Africa 16. Thailand 17. Tunisia 18. UK 19. USA 20. Uruguay	1. Argentina 2. Australia 3. Brazil 4. Canada 5. Germany 6. India 7. Japan 8. South Africa 9. Thailand 10. UK 11. USA 12. Uruguay	1. Argentina 2. Australia 3. Brazil 4. Canada 5. Germany 6. Greece 7. Japan 8. Romania 9. South Africa 10. Thailand 11. UK 12. USA 13. Uruguay	1. Australia 2. Germany 3. Japan

technologies, ART, IVF, sperm cryopreservation, embryo cryopreservation, oocyte cryopreservation, ovarian tissue cryopreservation, and testicular tissue cryopreservation. If providers were able to access one or more national, or multi-national registry, they were considered to have a registry available to them. Hospital level registries, local registries or regional registries were not recognized by this internet search, as we aimed to review registries available to larger populations on the national level. No additional national oncofertility registries were identified through this internet search. Therefore, the final list of countries around the globe that have official national oncofertility registries includes Australia, Austria, Germany, Japan, New Zealand, and Switzerland (Table 2). Some other countries such as the USA and Denmark are on their way to establish official national registries for oncofertility care. By reviewing such a global landscape, we highlight the urgent need for having a well-established official national oncofertility registry in each country to monitor oncofertility services in a way that best serves patients.

Establishing a national oncofertility registry is a very challenging process. A 2019 report by the leaders of *Ferti*PROTEKT, the Oncofertility Consortium and the Danish Fertility Preservation Network discussed the logistical considerations when initiating oncofertility networks, many of which should also be considered when establishing oncofertility registries (16). In the early stages of network development, it is crucial to consider structural details such as whether the oncofertility registry will be centralized or a collaborative effort of smaller networks with shared goals and responsibilities operating under the same guidelines. Governments, institutions and cryobanks may serve as the host and primary site of the oncofertility registry, depending on the intended size and structure of the collaborative effort. Estimates of population size and local differences which could influence care logistics (insurance, cultural differences, language, etc.) should be weighed when considering the inclusion of countries or regions. FertiPROTEKT, for example, is available to all German-speaking countries (Germany, Austria & Switzerland) (16). Consideration should also be given to selecting a list of data to be collected. Current oncofertility registries often include demographics such as age and gender, as well as health information such as the type and stage of the cancer diagnosed, type of fertility preservation services attempted, as well as measures of success (e.g.: cancer and fertility preservation outcomes, quantity of samples collected for cryopreservation, pregnancy rate, and live birth

TABLE 2 Countries around the globe that have official national oncofertility registries.

Country	Official National Oncofertility Registry	Website
Australia	Australasian Oncofertility Registry (AOFR)	http://www.futurefertility.com.au/registry/
Austria	FertiPROTEKT Network Registry	https://fertiprotekt.com/en/patients/
Germany	FertiPROTEKT Network Registry	https://fertiprotekt.com/en/patients/
Japan	Japan Oncofertility Registry (JOFR)	http://www.j-sfp.org/about/registry.html
New Zealand	Australasian Oncofertility Registry (AOFR)	http://www.futurefertility.com.au/registr/
Switzerland	FertiPROTEKT Network Registry	https://fertiprotekt.com/en/patients/

rate). Among current registries, there is variation in whether members are required to fill out all information, or if some of the information is voluntary- this too is a consideration (4). Providers may be concerned about finding time to input data. Therefore, it is recommended to keep required information to a minimum and enlist the support of other professionals such as patient navigators when completing such tasks.

Startup cost can be anticipated to be an early logistical concern. Various sources of funding may be considered, including research grants, government agencies, as well as private groups and societies. Located in Australia and New Zealand, the multisite Australasian Oncofertility Registry was established by The FUTuRE Fertility research team as part of a study. The registry includes information regarding referrals, uptakes, complications, and outcomes of oncofertility services (17). The group plans to compare data to other healthcare datasets to carry out additional studies and expand the clinical picture. Collectively, this data will inform evidence-based guidelines and resources. Other considerable startup costs include cost of website creation, establishing standard operating procedures for the recruitment of centers and standardization of data collection and deposition. In 2018, the Japan Society for Fertility Preservation (JSFP) launched the Japan Oncofertility Registry (JOFR). A recent article on JOFR showed that as of January 2022, over 7000 cases from more than 100 fertility centers have been registered in Japan. JOFR aims to keep disseminating information on cancer prognoses, pregnancy rates, and other oncofertility outcomes to help monitor and improve oncofertility services in Japan (18).

A known challenge in oncofertility is its multidisciplinary nature, as success requires close coordination between reproductive medicine specialists, reproductive biologists, and oncologists in various disciplines. One report suggests that approximately 20% of patients seeking oncofertility services sought advice independently, without the recommendation of their oncologist, highlighting the need for the encouragement of collaborative care (19). When determining leadership, representation from multiple specialties may be important in forming a foundation of cooperation that is necessary for long-term sustainability.

While this paper highlights the deficit of official national oncofertility registries around the globe, some limitations should be noted. While we evaluated the availability of registries, some registries are still under development and hence excluded from our analysis. Several registries are structured differently from each other, and we are yet to understand how such variability affects the success of these registries. Utilization and adherence to registration are necessary to consider when attempting to understand the success of registries, but this data was unavailable. Challenges in obtaining this data included language barriers, lack of publicly available data, and in some cases, obstructed ability to access information when viewed from outside of the host country.

5 Conclusion

According to our online pilot survey, responses from 20 countries were collected and showed that only 3 countries (Australia, Germany & Japan) have well-established official national oncofertility registries. The Australian official national oncofertility registry is part of Australasian Oncofertility Registry that also includes New Zealand. The German official national oncofertility registry is part of FertiPROTEKT Network Registry for German speaking countries that also includes Austria & Switzerland. The Japanese official national oncofertility registry includes Japan only and called Japan Oncofertility Registry (JOFR). A supplementary internet search confirmed the aforementioned results. Therefore, the final list of countries around the globe that have official national oncofertility registries includes Australia, Austria, Germany, Japan, New Zealand, and Switzerland. Some other countries such as the USA and Denmark are on their way to establish official national registries for oncofertility care.

Although oncofertility services are expanding globally, very few countries have well-established official national oncofertility registries. By reviewing such a global landscape, we highlight the urgent need for having a well-established official national oncofertility registry in each country to monitor oncofertility services in a way that best serves patients. We call for the creation of such registries, with consideration to the practical challenges in doing so especially the logistical and financial challenges.

Author contributions

All authors contributed equally in conceptualization, methodology, data collection and analysis, manuscript writing, reviewing & final editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Setup of a cryobank for ovarian tissue in a university-based setting

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Establishing and maintaining a newly set-up cryobank for ovarian tissue in a university setting requires at least 1 year's notice to start financial, spatial, lab equipment, and employee acquisition planning. Right before and after the start of the cryobank, the newly founded team should introduce itself to the hospitals and local and national health systems *via* mail, print flyers, and symposia in order to share the possibilities and the knowledge. Potential referrers should be provided with standard operating procedures and advice on getting used to the new system. Especially in the first year after the establishment, all procedures should be internally audited in order to avoid possible difficulties.

KEYWORDS

cryopreservation, freezing, gonadotoxic, children, female, infertility

Introduction

Establishing a cryobank for ovarian tissue (OT) that is technically state-of-the-art in terms of both personnel and equipment is the basic prerequisite for establishing a new medical unit at a German university hospital. This certainly requires good planning and a certain amount of lead time in order to fulfill the aforementioned prerequisites. Once these conditions have been met, however, the real work begins, because now the newly installed cryobank team must ensure that a wide variety of target groups are informed that there is now a cryobank at this location and what specific services are available for patients and medical staff.

Planning of the setup of a centralized cryobank and financial aspects

Because of the few existing cryobanks for fertility preservation (FP) in Germany at the moment and the unproblematic dispatch of the cryomaterial, it is indispensable to engage with and become known to groups far beyond the normal catchment area. Prior to the start of a cryobank, there were many meetings with the head of the department of OB/GYN, the

dean of the medical faculty, and the legal and the financial departments, which dealt with spatial possibilities, financial aspects, the design of the contracts, the scientific foci of the university hospital, and obviously the staff needed. Either new or existing staff with experience in the *in vitro* fertilization (IVF) lab or newly employed experts in the field were qualified for this position. In our case, meetings with the financial department were more fruitful as an IVF unit has been established before. Furthermore, next to the lab and storage space for the cryobank, an area for consultations should be offered within the center in the IVF unit, representing specialists in reproductive medicine (Supplementary Figure 1). The minimal equipment of a cryobank should be a laminar flow for the different steps during freezing and thawing, a fridge and a refrigerator for the transport and cryopreservation medium, a printer for the cryobonds, a slow freezing unit with access to liquid nitrogen, and a storage tank. As part of the public health system, the financial benefit could be more nonprofit than established as a private cryobank. A private cryobank for ovarian tissue cryopreservation (OTC) in Germany would need about 350,000€/year for its establishment and maintenance, which is more expensive than that estimated by Kyono et al. (1). In our unit, the cryobank started on the same campus in the GCP unit, focusing on blood stem cells and cord blood. In order to offer national support, numerous transport boxes and cooling elements have been bought, and a contract with a parcel service has been signed (Figures 1A, B). Regarding the number of staff needed on site, a minimum of two specialists should be educated to freeze and thaw OT, supported by a patient manager who responds to the patient's questions and concerns and organizes the logistics of the boxes and the data management. About 400 patients a year perform OTC, according to the Fertiprotekt data collection and data from Japan (1, 2). The patient's burden to pay for FP, including OTC, will be covered by the medical insurance in Germany soon. Since 2021, the costs of freezing mature oocytes for FP are covered by the insurance companies as long as the patient is over 18 years old and, in the case of breast cancer, hormone receptor negative.

Information on referrers and potential patients

A distinction must be made here as to who the information is intended for. On the one hand, there are affected patients of reproductive age or even children with a malignant disease who will be treated with chemotherapy and/or radiation and who may be at risk of moderate to complete loss of fertility, depending on the treatment regimen (3, 4). In addition, there are patients with benign diseases, e.g., thalassämia, who are also going to receive a gonadotoxic treatment (5). Patients with genetic predisposition or severe endometriosis disease may also be considered for fertility preservation (5). All patients, especially those with an oncological disease, are already focused on treatment and survival. Due to the absence or limited possibilities for biological parenthood after successful therapy and remission for those receiving high-risk therapy, they should nevertheless be given the opportunity to consider the topic of FP. Here, the attending physician or specialist is certainly the one to provide the patient with information about FP or the address and/or contact of a specialized counseling unit. In Germany, the documentation of the patient's education concerning FP is part of the certification prerequisites for cancer centers.

Moreover, gaining information through an Internet search is also becoming increasingly important nowadays. Here, patients inform themselves independently, so it is important to initiate the

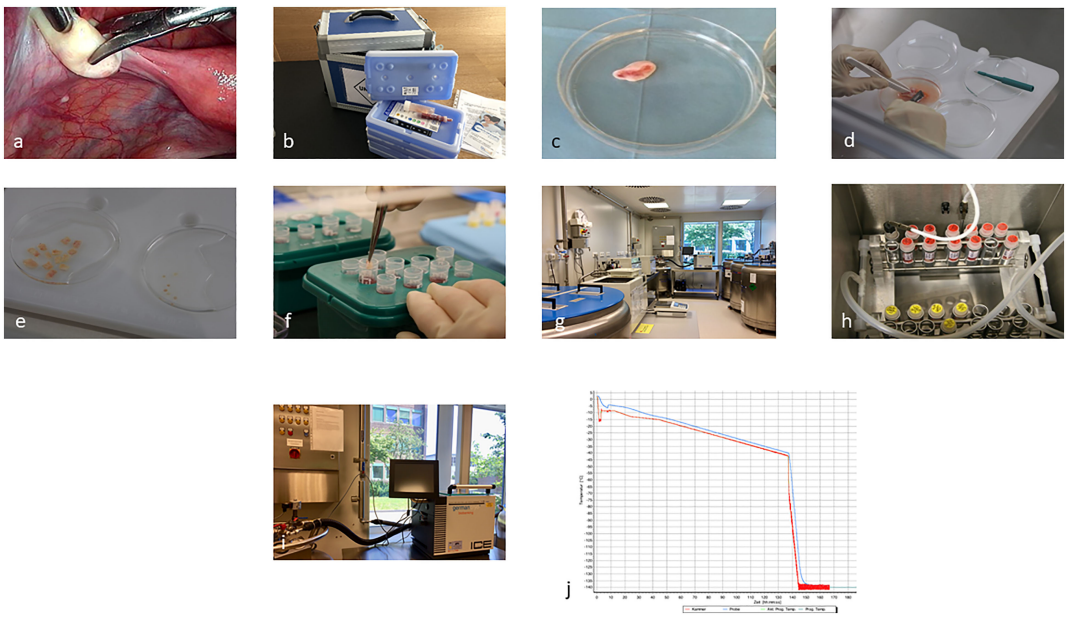

FIGURE 1
Representing the process from laparoscopic biopsy **(A)**, (overnight) transport **(B)**, and preparation and freezing of the cortical biopsies **(C–J)**.

media presence at the same time during the establishment of a cryobank. An informative and easy-to-navigate website with information about the existing options, information on contact (in addition to phone contact, electronic contact is increasingly favored because this is independent of the opening hours of the center and is therefore considered a very low-threshold offer), and, in the case of the OTC and or gametes, also information on the costs and associated legally regulated requirements for cost coverage by statutory health insurance, plays an immense role. Closely related to web performance is good accessibility. This requires specially trained personnel on the phone and, if possible, an individual phone and/or mobile number that is used only for matters of fertility-preserving needs to guarantee short-term help.

Also, foundations (Deutsche Stiftung für junge Erwachsene mit Krebs, Stiftung Lebensblicke), associations, and self-help groups (Jung und Krebs e.V., Krebs-Selbsthilfe, Deutsche Krebshilfe, Frauenselbsthilfe Krebs-Bundesverband e.V., BRCA-Netzwerk e.V.-Hilfe bei familiären Krebserkrankungen, Rheuma-Liga, Endometriose Vereinigung) are playing an important role in which emotional support is offered as well as knowledge about a disease, treatment strategies, and often also information around support, applications, etc. Therefore, it makes sense for the cryobank team to introduce itself to the local foundations/self-help groups, as well as to national ones, so that they are precisely informed about the technical possibilities and options. Members of the foundations or self-help groups can then pass this knowledge safely, quickly, and objectively to those affected. It also makes sense to keep on giving presentations at patient information days (e.g., breast cancer day), at the citizens' university, or at meetings of self-help groups and associations, as information can also be lost there due to personnel changes.

A very current approach, which is also desirable in the area of FP, is represented by the project of the so-called professional patients or patient coaches, which has been initiated at our university hospital with a special focus on oncological patients. Here, formerly ill patients act as on-site advisors for those currently seeking help and navigate them not only physically through the often jungle-like wounded localities in the clinics but also in terms of content. The information and detailed education of these patient coaches are also good multipliers for the flow of information to the patient.

In addition to patient-oriented information and the right information tools and flow for them, the training of medical professional groups is important (Supplementary Figure 1). Here, it must not be forgotten that not only medical personnel who care for patients with malignant diseases but also those who care for patients with benign diseases such as immune diseases and endometriosis, as well as those with genetic predispositions such as Turner syndrome, receive differentiated and, if possible, regular information through lectures, mails, and events on the topic from colleagues on site or in the area of the cryobank. Especially when a cryobank is newly established at a location, it often takes longer for this information to get around, so it is good to organize information events even before and during the cryobank's opening to report on this increase in possibilities. From our own experience, we can state that the sole information available through clinic news, letters, and emails is not sufficient to raise an awareness of the new situation, but the personal approach has a much higher value. Especially because the field of FP is very fast developing and therefore changing, personal and regular information of all involved specialties provides a solid counseling offer according to the current S2k guideline (6). In order to meet the needs of the healthcare providers and to gain an update on the routine of cryobanks, several questionnaires have been created in the last decade emphasizing the need for specialists and best care for all patients independent from financial burdens or social impacts (7–10).

The daily routine of a cryobank, including thawing and transplantation service

Surgery and freezing of OTC

Immediately prior to the start of therapy, OT can be obtained by laparoscopy within 1–2 days after counseling or even the diagnosis of a malignant or benign disease (Figures 1A-C). All follicles are located in the OT cortex, which is dissected into at least 10 equal-sized pieces of tissue and then cooled in a controlled manner using a computer-controlled program until it is completely frozen to −140° C (11, 12) (Figures 1C-J). The frozen samples are then transferred directly to the cryostorage container, where they can be stored indefinitely in the nitrogen vapor phase. In our cryobank, the initial storage period according to the contract with the patient, is 1 year, which is then extended annually. In Germany, there are only three centralized cryobank and three to five smaller centers offering processing, cryopreservation, and permanent storage of OT. Only the centralized cryobanks offer overnight shipping of OT nationwide (13). Only the centralized facilities have the special equipment and trained personnel to ensure a standardized good quality, which is needed in terms of transplantation after the patient's recovery from the disease and the wish to conceive (14, 15).

The cryobank and the clinic where the laparoscopy takes place have signed a cooperation contract and a delimitation of responsibility agreement containing the responsibilities for processing, cryopreservation and storage, and presurgery, surgery, and postsurgery measures. The goal is always to provide the patient with the best possible care and service. A close exchange between the cryobank and the patient's clinic is very important, including the registration of a patient during or after counseling and the coordination of the shipment of the boxes for transport (13, 16, 17) (Figure 1B). Essential for the information of the staff on-site as well as in external clinics is a standardized procedure, which is defined in a standard operating procedure (SOP) (Supplementary Figure 1). This then applies to all employees of the collection clinic, the cryobank, as well as the gynecological outpatient clinic/ward, patient management as well as the OR, and consequently all persons involved in the OT collection, preparation, and storage. The persons responsible for the various tasks should be named in the SOPs with their contact data and should receive regular training.

Annual random internal audits are required. A physician is responsible for the initial interview with the potential patient, including the patient's history, which is also possible *via* e-consult in terms of a pandemic, for example (10). The patient must be informed and advised about the expected gonadotoxicity of the therapy and the different options for FP (Supplementary Figure 1). Basic gynecological sonography, blood sampling for AMH, and serology for possible infectious diseases must be performed before the laparoscopy is scheduled. The cryobank provides the collection clinic with all relevant documents, such as patient information on cryopreservation, a contract, and the cost agreement (Supplementary Figure 1). The referring external clinics receive a checklist from the cryobank (Supplementary Figure 1). During the counseling about the partial/complete OT removal by laparoscopy, a possible transposition of the ovaries and potential contraindications, as well as a combination with port access, must be clarified. Important for the processing and storage of the OT in the cryobank are negative results concerning the infection parameters that need to be present 7 days before surgery. According to the German Medicinal Product Act, the Tissue Act, and the Pharmaceuticals and Active Agent Manufacturing and Distribution Ordinance, this includes anti-HIV ½ p24 antigen, HBs-Ag, anti-HBc (in case of positivity: HBV-PCR), anti-HCV-IgG, TPHA, and in case of stay in the ZIKA area in the last 6 months, the ZikaV-IgG and ZikaV-IgM. On the day of the surgery, it is the responsibility of the clinic to check the records for completeness. The cryobank checks the consent form for cryopreservation and cost coverage on the day of arrival. On the day of the surgery, a special transport box is provided by the cryobank with appropriate transport medium and nonfrozen refrigeration units (Figure 1B). The transport temperature should be between 4° and 8°C, and the transport time should not be more than 24 h (13). The surgeon must be trained to avoid an ovary with an active corpus luteum. If no corpus luteum is present, partial or complete removal of the ovary is preferred on the left side in Germany, preferably in one piece (Figure 1C) (15, 18). Any additional slicing of the ovary should be avoided, as should coagulation. Follicles are extremely temperature-sensitive to higher temperatures than 37°C. Therefore, coagulation will destroy several millimeters of the OT cortex, resulting in the loss of the embedded follicles. The removed ovary is placed directly into the cooled transport medium. Custodiol ® (Dr.Franz Köhler Chemie GmbH, Bensheim, Germany) is used as our medium of choice. This organ perfusion medium is pH-stable and prevents ischemic processes in the tissue, even after 24 h at 4°C. Furthermore, a biopsy of the ovary must be sent to the pathology department of the clinic. The cryobank collects information about the surgery and, later, the results from the pathologist. Once the ovary is removed and embedded in Custodiol®, the temperature should remain between 4°C and 8°C, including the processing of the tissue (Figures 1C-F). The preparation of the ovary is performed under sterile conditions under laminar flow. It is important to avoid contamination of the OT during storage and later transplantation. The ovary section is processed in fresh Custodiol® with sterile forceps, scalpels, and biopsy punch under laminar flow in a 100-mm dish on a cooling plate (Figure 1D). The stroma of the ovary is removed by scratching with a scalpel (Figure 1D). The goal of the dissection is to expose the cortex as isolated as possible, without portions of blood vessels, corpus luteum, or other tissues (11). Rectangular pieces 5–10 × 3–6 mm in length are cut from the cortex (Figure 1E). After completion of the dissection, all pieces are equilibrated in a sterile vessel with 10 ml of cryomedium. Currently, cryomedium is not commercially available. The formulation we use for the freezing process of the OT corresponds to the composition of Roger Gosden's medium. In order to assess the vitality of the tissue before freezing and after subsequent thawing before transplantation, 3 × 2-mm biopsies are obtained by punching from different regions of the cortex (Figures 1E, Supplementary Figure 2). The so-called vitality test is performed after digestion with collagenase and subsequent incubation with calcein (Thermo Fisher Scientific, Meerbusch, Germany). The vital follicles can be examined by fluorescence microscopy (19) (Supplementary Figure 2). The entire freezing process with a slow freezing method and automatically induced seeding takes about 2.5 h and is recorded (Figures 1G-J). Samples are first progressively cooled to −40°C and then to −140°C before being transferred to liquid nitrogen for long-term storage. In an electronic management system for the cryopreserved materials, an overview of all incoming and outgoing samples can be obtained.

Thawing service

At our location, we have also established the offer of the so-called mobile thawing service for OT, so that the respective clinic, which has removed a tissue, can transplant it later (17). Nevertheless, most transplant patients are cared for in a few specialized clinics with well-trained surgeons. The date of the OT is organized by the respective clinic, the patient, with the oncologist's informed consent, and the cryobank. A sterile hood for the thawing process should be available within a 10-min walk from the surgery theater. The well-trained staff of the cryobank and the frozen patients' tissues move to the respective clinic. The thawing process takes about 60 min. All media and technical devices are transported as well. Before slicing the peritoneal pocket, the fallopian tubes are checked for perturbation to enable spontaneous pregnancy. The transplantation in a peritoneal pocket and the thawing procedure are coordinated in a timely manner (Figures 2A-D) so that the ovarian grafts are transplanted as soon as possible after thawing. Previously, the grafts were orientated with the outer cortex toward the tube. Recently, Kristensen et al. reported that revascularization does not need this orientation, which reduces the time of the surgery as well (20).

Discussion

For the establishment of a cryobank for OT in a university setting, some prerequisites need to be fulfilled: lead time, well-educated and dedicated staff, on-site space, and a financial plan covering the costs of the establishment and the possible outlay for the first 5 years. A major strength of the Faculty of Medicine at the

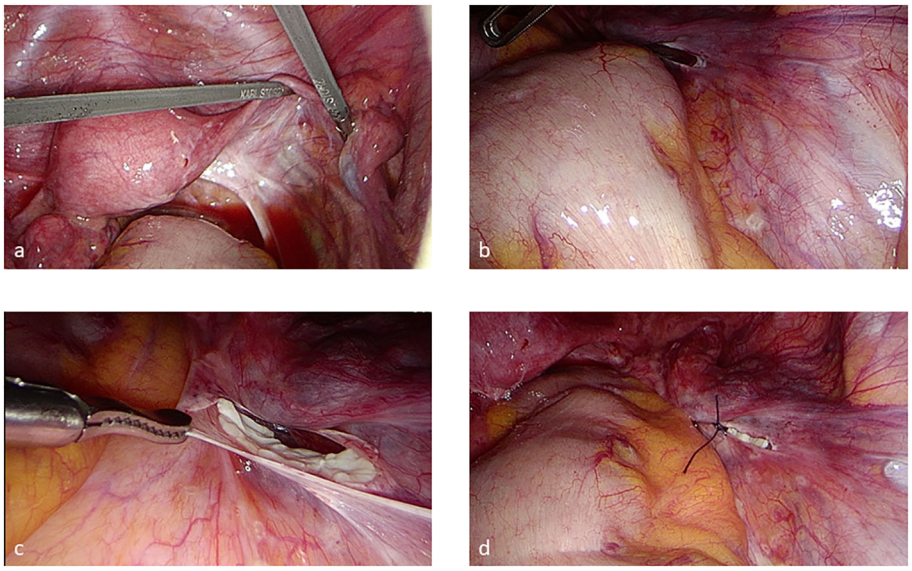

FIGURE 2
Transplantation in a peritoneal pocket by laparoscopy **(A–D)** after chromopertubation. The pieces should be transplanted on the right side if this is possible to simplify ovum pick-up if the patient needs an ART treatment.

UKD lies in the interest in and the priority research field of oncology and the establishment of state-of-the-art treatment options. Since 2013, the University Tumor Center (UTZ) has been certified as an Oncology Center of the German Cancer Society (Deutsche Krebsgesellschaft (DKG)), and from 2014, oncology at the university hospital was funded as a top center of the German Cancer Aid (Deutsche Krebshilfe). Furthermore, the Düsseldorf School of Oncology (DSO) supports clinical education and translational research in different disciplines, e.g., hematology and oncology, pediatrics, urology, and gynecology. Within the framework of the Centre for Integrated Oncology (CIO^{ABCD}—Aachen, Bonn, Cologne, and Düsseldorf), the four sites collaborate intensively to improve patient care using combined and personalized approaches, e.g., molecular tumor boards. In order to advance treatment success and care for the future, FP for patients of reproductive age as well as children is part of the quality assessment of the DKG. We aim to cross interdisciplinary and regional boundaries through education and knowledge exchange in order to improve patient care. We would like to strengthen and extend innovative, interdisciplinary clinical and scientific training structures and platforms to facilitate a novel holistic view of FP concepts, including more than oncologic disciplines, e.g., ethics, psychosomatics, public health, and law.

A culture of staff working to their full potential and treating patients with compassion and understanding is very important during this stressful time for the patients and their relatives. Due to the recurring ethical and legal complexities of diverse aspects of FP (e.g., reproductive autonomy, trans/nonbinary person, cost coverage), there has also been close cooperation with the institutes of ethics and the law school for many years. Concerning quality assessment, it might be an option to design a survey for the patients who cryopreserved and stored their tissue in our cryobank (21). Furthermore, referrals can be actively surveyed, e.g., every 2 years, in order to optimize the service of the cryobank. Possible foci might be the availability *via* mail or phone, the costs, and the design of the flyers.

Conclusion

The development of a corporate professional identity is an essential instrument in the healthcare environment in order to create a positive and unique impression on the patient and the referring professionals. Nowadays, the patient has a wide range of options in outpatient care available to them compared to former times when the first baby was born after freezing and thawing of OT (22). This has also changed the role of the physician in society. The physician now has to develop organizational and business abilities in addition to providing advice, care, and healing for the patient. Hence, the importance of translation and transfer from basic research to clinical application and comprehensive healthcare will continue to increase and should improve patient care worldwide.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

DB-B directed the project; DB-B, IS, and AB wrote the article. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1193178/full#supplementary-material

SUPPLEMENTARY FIGURE 1
A friendly environment and a sufficient timeslot in the schedule should be offered for counseling.

SUPPLEMENTARY FIGURE 2
Vitality testing using immunofluorescence staining with calcein **(A, B)**. **(C–E)** Images of follicles with different sizes (primary to secondary follicles). The shallow green dots represent already atretic follicles in the biopsy.

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Ovarian stimulation and oocyte cryopreservation in females and transgender males aged 18 years or less: a systematic review

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Background: Fertility preservation is an important healthcare focus in the paediatric and adolescent population when gonadotoxic treatments are required. Ovarian stimulation (OS) resulting in oocyte cryopreservation is a well-established fertility preservation option in the adult population. It's utility, however, is little known in young patients. The purpose of this review was to synthesise the available literature on OS in patients ≤18 years old, to identify gaps in current research and provide suggestions for future research directions.

Methods: Using PRISMA guidelines, a systematic review of the literature was performed for all relevant full-text articles published in English in Medline, Embase, the Cochrane Library and Google Scholar databases. The search strategy used a combination of subject headings and generic terms related to the study topic and population. Two reviewers independently screened studies for eligibility, extracted data and assessed the risk of bias. Characteristics of the studies, objectives and key findings were extracted and summarised in a narrative synthesis.

Results: Database search and manual review identified 922 studies, 899 were eliminated based on defined exclusion criteria. Twenty-three studies were included and comprised 468 participants aged ≤18 years who underwent OS (median 15.2, range 7-18 years old). Only three patients were premenarchal, and four patients were on treatment to suppress puberty. Patients had OS for a broad range of indications including oncology treatment, transgender care and Turner syndrome. A total of 488 cycles of OS were completed, with all but 18 of these cycles (96.3%) successfully resulting in cryopreserved mature oocytes (median 10 oocytes, range 0-35). Fifty-three cycles (9.8%) were cancelled. Complications were rare (<1%). One pregnancy was reported from a female who had OS aged 17 years old.

Conclusion: This systematic review demonstrates that OS and oocyte cryopreservation is achievable in young females however there are only a few cases in the literature describing OS in premenarcheal children or those who have suppressed puberty. There is little proof that OS can lead to pregnancy in adolescents, and no proof that this can be achieved in premenarchal girls. Therefore it should be regarded as an innovative procedure for adolescents and experimental for premenarcheal girls.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=265705, identifier CRD42021265705.

KEYWORDS

oocyte cryopreservation, fertility preservation, ovarian hyperstimulation, ovarian stimulation (OS), paediatric and adolescent gynaecology, oocyteretrieval, oncofertility, paediatric oncofertility

1 Introduction

Fertility preservation is now an important component of healthcare in the paediatric and adolescent population where treatment involves risk to future fertility, most commonly because of administration of gonadotoxic agents (1). Therapies for cancer, rheumatological or haematological diseases, and for gender dysphoria, may be detrimental to the ovary at any age (2). Similarly, a range of genetic conditions, most prominently Turner syndrome (TS), may result in premature ovarian insufficiency at an early age. Future infertility is a significant source of concern and anxiety for both a young patient and their family members in these circumstances (1).

Oncofertility services are developing rapidly around the globe to support those at risk of treatment-related infertility and assist with fertility preservation in a timely manner (3). Therapies to protect or restore fertility are well established in the adult female population (2, 4, 5); however, data and options are limited in the paediatric and adolescent population. Clinicians may find it challenging to discuss and offer invasive fertility preservation treatments to young people with little data on proven long-term benefit (6, 7).

For many years clinicians have used ovarian shielding, transposition away from the radiation field, and GnRH analogues in an attempt to protect fertility, which have conflicting or scarce evidence of benefit, particularly in minors (8, 9). More modern fertility preservation options include ovarian tissue cryopreservation (OTC), *in vitro* maturation (IVM) and ovarian stimulation (OS) for oocyte cryopreservation (2, 10).

Until very recently ovarian tissue cryopreservation has been the only assisted reproduction technology (ART) offered for pre-pubertal girls and post-pubertal females where there is limited time before cancer treatment (11). It is considered an established procedure in adult women with around 200 births reported to date, but so far, there have been only 2 live births from premenarcheal tissue (12, 13). IVM involves retrieval of immature oocytes from ovaries after minimal or no gonadotrophin stimulation and their subsequent maturation in the laboratory. In the context of fertility preservation, collection of immature oocytes from adult ovarian tissue and IVM is experimental and very few livebirths have been reported (14). OS resulting in oocyte or embryo cryopreservation is the most successful form of fertility preservation for biological females (15), however, it has been studied mainly in adult populations (16). Additionally, there are questions around oocyte quality in very young women, as studies of follicle morphology have demonstrated an increase in abnormal types in the young (17). Embryo cryopreservation poses ethical issues in the young and may prove limiting in the event of partner change (18).

Given that many patients will only have one opportunity to preserve fertility prior to commencing gonadotoxic treatment, it is important that they are offered preservation options that will give them the greatest chance to achieve future parenthood. There are reasons why oocyte cryopreservation may be considered an addition to, or preferred to OTC in selected populations. A single stimulation cycle followed by a minimally invasive oocyte retrieval, compared with laparoscopy and its associated recovery for OTC, may make the procedure more acceptable to some patients (19). The possibility of reintroducing malignant cells in patients diagnosed with haematological cancers (3, 20) means that reimplantation of untreated ovarian tissue may not be considered safe in some cases. In patients with genetic conditions with increased risk of premature ovarian insufficiency where the pathology is intrinsic to the ovary, such as TS, the accelerated germ cell loss with thawing and ovarian transplantation has led to uncertainty about the likely success of ovarian tissue reimplantation (21).

The aim of this systematic review is to evaluate oocyte cryopreservation, by means of OS in the paediatric and adolescent population. We compare age, diagnosis and pubertal and menarchal status and comment on success rates, adverse outcomes, and psychological morbidity. Additionally, we identify future research directions that may support the successful adoption of these therapies around the world.

2 Methods

2.1 Search strategy

In accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, a systematic search of the literature was performed for all relevant full-text articles published in English in Medline, Embase, the Cochrane Library, and Google Scholar databases (PROSPERO registration number CRD42021265705).

The following search terms were used in different combinations: "ovarian stimulation", "oocyte cryopreservation", "*in vitro* fertilization (IVF)", "fertility preservation" (see Supplementary Material for all the search terms and search strategy). A final search was conducted on 14/08/2022 to ensure inclusion of all relevant studies.

2.2 Study selection

Articles were included if they reported on any clinical outcomes of oocyte cryopreservation in the paediatric and adolescent (≤18 years old) population. Studies that included patients with other fertility preservation procedures were included if data for the individual subtypes of fertility preservation procedures were reported separately. Studies that only described alternative fertility preservation options or reported on outcomes in those >18 years old were excluded.

Case series, prospective and retrospective comparative cohorts, controlled (non-randomised) and randomised controlled trials, review articles, cross-sectional studies, and case reports were included. Guidelines, commentaries, conference abstracts, and pilot study data that were also reported in a published study already included in the review were excluded.

References (n=922) were imported into a Covidence database where duplicates were removed. The remaining abstracts (n = 730) were subsequently reviewed independently by two authors (MS, KM) and all those describing outcomes of COS or oocyte cryopreservation in females 18 years or younger underwent full text review. Based on title and abstract screening, 663 articles were excluded, 67 full text articles were assessed for eligibility and as 5 articles were not accessible, 62 were eligible for review (Figure 1).

2.3 Data extraction and analysis

Data from articles were extracted into a pre-designed database. Year of publication, country of study, study objectives, study design, sample size, patient characteristics, intervention, outcome measures, and findings were documented. No relevant outcomes were found for 32 articles and 7 articles did not discuss the relevant population and were therefore excluded. The remaining 23 studies were included for systematic review. No discrepancies were found, therefore a third reviewer was not required for definitive decisions on the data extraction.

The two independent reviewers performed methodological quality assessment for each study. Due to the range of study designs being analysed, Qualsyst (Appendices 1, 2) was used to

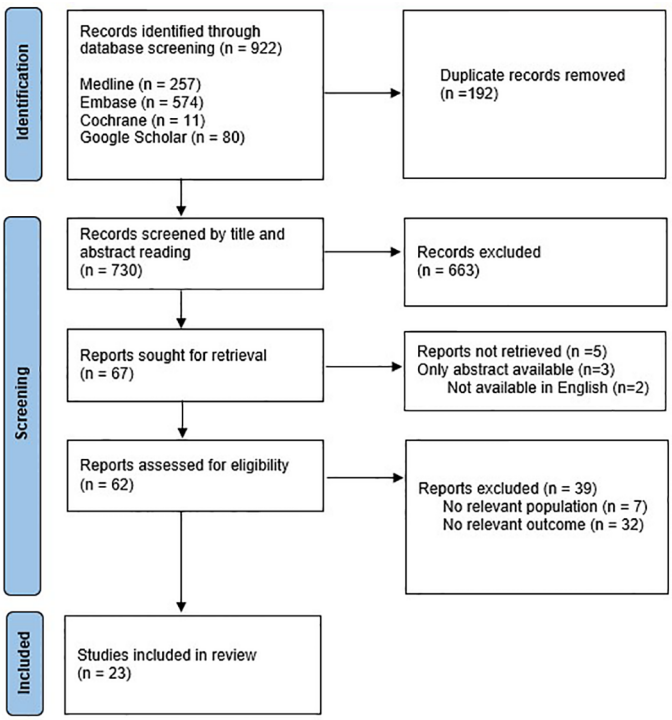

FIGURE 1
Identification of studies *via* databases and registers (22).

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facilitate the assessment of risk of bias for each study. Each study received a percentage score. Any discrepancy was resolved through discussion.

We attempted to correspond with some study investigators to resolve data queries and request additional data as required regarding undocumented pubertal or menarchal status, side effects to treatments, or sub analysis of age groups, and included relevant additional information provided.

3 Results

3.1 Study characteristics

The 23 papers reviewed were from USA (n=16), UK (n=3), Israel (n=2), Sweden (n=1), and China (n=1). They include case reports (n=10), case series (n=6), retrospective cohort studies (n=4), prospective cohort studies (n=2), and a letter to the editor describing a case report (n=1) (Table 1) (10, 19, 23–43).

The studies included 468 participants who underwent OS (median age 15.2 years, range 7-18) with a total of 488 cycles of OS completed. All but 18 of these cycles (96.3%) successfully resulted in mature oocyte cryopreservation.

3.2 Outcomes according to age

The four large cohort studies in this review (30, 41–43) described a total of 404 participants ≤ 18 years as grouped data, and did not provide a breakdown of outcomes in relation to age category or Tanner stage. Across the remaining 19 studies, 64 participants with a median age of 15 years (range 7-18) were described (Table 1).

There were three case reports of OS in premenarcheal children, one of whom was prepubertal (10). The prepubertal patient was a 7-year-old with mosaic TS (45,X[37]/47,XXX[15]) who initially underwent OS with gonadotropin-releasing hormone agonist (GnRHa) trigger which failed to yield oocytes. The second cycle with hCG trigger was successful, resulting in the retrieval of six oocytes and cryopreservation of all six mature oocytes. Martel et al. (39) described a 14-year-old premenarchal patient with TS who froze two oocytes over one cycle, using an hCG trigger. Her pubertal status was unknown. Reichman et al. (31) described a 13 year old premenarchal peri-pubertal (Tanner 3 breast and Tanner 1 pubic hair development) with myelodysplastic syndrome. An hCG trigger was used for this patient, and 18 mature oocytes were cryopreserved in one cycle, before gonadotoxic treatment commenced.

Another notably young patient ≤ 12 years-old (their exact age was not specified) was a transgender male who had 9 mature oocytes cryopreserved over 2 cycles (29). A further 31 patients aged 13-15 years old (10, 19, 26–29, 31, 32, 35, 36, 38–40) cryopreserved a median of 9.5 oocytes (range 0-22) and 31 patients aged 16-18 years old (19, 23–25, 27–29, 33, 34, 37, 39) cryopreserved a median of 14 oocytes (range 0-35).

A multi-center cohort study that assessed OS in oncology and non-oncology populations, compared outcomes in those aged 13 to

TABLE 1 Description of studies examining age, pubertal and menarchal status, diagnosis, ovarian reserve testing, and oocytes retrieved and cryopreserved (see Supplementary Material for more detailed results in table format).

Author, Country	Diagnosis	Study design	Age (years) median (range)	Pubertal status	Ovarian reserve testing: median (range)[#]			Controlled ovarian stimulation protocol, trigger	Oocytes retrieved, median (range)[2]	Oocytes cryopreserved, median (range)[◊]
					AMH ng/mL (n=52)	FSH mIU/mL (n=33)	AFC (n=25)			
Maxwell et al, USA (23)	TG male	Case Report	17	PM				Antagonist cycle	21	17
Rothenberg et al, USA (24)	TG male	Case Report	16	Pubertal suppression (GnRHa at 14 years, Tanner II)		0.89		GnRHa (never ceased), rFSH + hCG, -, hCG trigger	5	4
Wallace et al, USA (25)	TG male	Case Report	17	PM	3.5	5.7	40	rFSH + hMG, antagonist, GnRHa and hCG trigger	39	35

(Continued)

TABLE 1 Continued

Author, Country	Diagnosis	Study design	Age (years) median (range)	Pubertal status	Ovarian reserve testing: median (range)[#]			Controlled ovarian stimulation protocol, trigger	Oocytes retrieved, median (range)[2]	Oocytes cryopreserved, median (range)[◊]
					AMH ng/mL (n=52)	FSH mIU/mL (n=33)	AFC (n=25)			
Martin et al, USA (26)	TG males	Case Report	15	Pubertal suppression (GnRHa at 10 years, Tanner II)	2.62	3.6	25	GnRHa, letrozole. rFSH + HMG, antagonist, hCG trigger	36	22
Insogna et al, USA (27)	TG males	Case Series (n=3)	15 (15-17)	PM (case 1 used GnRHa at 12)	3.1 (0.9-5.29)			hMG, hCG trigger/rFSH + hMG, antagonist, GnRHa trigger/rFSH + hMG, antagonist, HcG or GnRHa trigger	20 (15-31)	12 (10-18)
Chen et al, USA (28)	TG males	Case Series (n=5)	16 (14-18)	PM	5.9 (3.6-6.5)			rFSH ± hMG, antagonist, hCG trigger	19 (11-28)	13 (8-25)
Barrett et al, USA (29)	TG males	Case Series (n=17)	- (12-18)	-(case 2 - Pubertal suppression with GnRHa)	3.18 (0.44-12.87)	5.65 (1.7-9.5)		rFHS, hMG or both, GnRH antagonist protocol or low-dose down-regulation protocol with GnRHa, hCG or GnRHa trigger	22 (5-43)	14.5 (3-26)
Amir et al, Israel (30)	(i)TG males; (ii) Females with cancer	Retrospective Study (n=48)	- (13-18)	PM		TG male: mean 5.4 ± 1.7 Female: -	TG male: mean 19.8 ± 5.6	rFSH, antagonist, GnRHa or hCG or dual triggers	TG male: mean 30.6 ± 12.8 female: mean 22 ± 13.2	TG male: mean 25.6 ± 12.9 female: mean 18.8 ± 11.2
Reichman et al, USA (31)	MDS	Case Report	13	Peripubertal, Premenarcheal	0.95	5.0	9	hMG, antagonist. hCG trigger	20	18
Peddie et al, UK (32)	MDS	Case Report	14	PM		7.1	17	rFSH + hMG, antagonist, hCG trigger	13	12
Cai, H et al, China (33)	MDS	Case Report	17	PM		3.27	7	rFSH, GnRHa trigger.	17	13
Tsampras et al, UK (34)	Aplastic anaemia, MDS	Prospective Study (n=2)	17, 17	–	7.34,8.01^		20,19	Gonadotropins, antagonist, hCG trigger. DuoStim	2 cycles: 21 (20-22)	2 cycles: 17 (13-21)
Garg et al, USA (35)	Hodgkin's Lymphoma	Case Report	14	PM	0.4		11	rFSH + hMG, antagonist, hCG trigger	13	11
Kutteh et al, USA (36)	Medulloblastoma	Case Series (n=3)	14 (13-15)	PM	3.61 (1.96-3.83)^		25 (15-31)	hMG, antagonist, GnRHa trigger	25 (18-26)	17 (12-23)
Kim at al, USA (37)	Pulmonary Hypertension	Case Report	17	–				Long agonist protocol rFSH and hMG	14	14

(Continued)

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TABLE 1 Continued

Author, Country	Diagnosis	Study design	Age (years) median (range)	Pubertal status	Ovarian reserve testing: median (range)[#]			Controlled ovarian stimulation protocol, trigger	Oocytes retrieved, median (range)[2]	Oocytes cryopreserved, median (range)[◊]
					AMH ng/mL (n=52)	FSH mIU/mL (n=33)	AFC (n=25)			
Lavery et al, UK (19)	Sickle Cell Disease	Case Series (n=8)	16 (14-18)	PM	10.6 (7.1-10.7)[^] n=5 missing	4.25 (1.2-7.6)	16 (6-20)	rFSH. Combination of agonist and antagonist protocols. HCG or GnRHa trigger	10.5 (5-31)	9 (1-30)
Oktay et al, USA (38)	TS mosaicism	Case Report	14	PM	0.9, 1.7	5.3		rFSH, antagonist, GnRHa trigger/rFSH + hMG, antagonist, GnRHa trigger	2 cycles: 9 (7-11)	2 cycles: 6 (4-8)
Azem et al, Israel (10)	TS mosaicism	Case Report	7	Prepubertal	1.13	5.2	5, 3	rFSH & rLH. GnRHa trigger/rFSH + rLH. HCG trigger	2 cycles: 3 (0-6)	2 cycles: 3 (0-6)
Martel et al, USA (39)	TS or TS mosaicism, 47 XXX	Case Series (n=11)	15 (13-18)	Variation	1.04 (< 0.003-2.99) n=4 missing	36.65 (5.2-74) n= 7 missing		"gonadotropins", antagonist, hCG ± GnRHa	1 cycle: 14 (0-21) 2 cycles: 0 (0-22) 3 cycles: 9.5 (0-19) 9 cycles: 3	1 cycle: 12 (0-16) 2 cycles: 0 (0-16) 3 cycles: 4 (0-8) 9 cycles: 0
Oktay et al, USA (40)	TS mosaicism, malignancy	Retrospective Study (n=4)[+]	13.5 (13-15)	PM	1.3 (0.76-1.6)	5.65 (5.6-7.8)	6 (5-11)	rFSH/hFSH + hMG or rLH. Antagonist, rHCG/hHCG/GnRHa triggers.	17.5 (8-21)	8 (4-10)
Hipp et al, USA (41)	Variety of diagnoses*	Retrospective Study (n=306)	- (≤18)	PM				In all age groups the most common stimulation protocol was antagonist		
Rodriguez-Wallberg et al, Sweden (42)	Variety of diagnoses**	Prospective Study (n=24)	16 (14-17)	PM				Not described	–	
Manuel, et al, USA (43)	Variety of diagnoses***	Retrospective Study (n=26)	16.5 (13-18)	–	2.72 (0.25-6.50)			rFSH +/- hMG, antagonist, hCG trigger.	13 (4-31)	10 (0-25)
Totals			15.2 (7-18)		2.9 (0.003-12.9)	4.5 (<0.1-20.5)	16 (5-35).		17 (0-43)	10 (0-35)

PM, post menarcheal; FSH, follicle stimulating hormone; AMH, anti mullerian hormone; AFC, antral follicle count; TG, transgender; MDS, myelodysplastic syndrome; rFSH, recombinant human follicle-stimulating hormone; GnRHa, gonadotropin releasing hormone agonist; rHCG, recombinant human chorionic gonadotropin; hMG, human menopausal gonadotropin; HCG, human chorionic gonadotropin; rLH, recombinant LH.

- = not described.

[#]Denominator changes due to missing data.

[◊] 1 cycle unless otherwise specified.

[^] AMH was converted to ng/mL for consistency.

[+] One case was duplicate, described in Oktay, et al. (38).

* cancer, aplastic anaemia, sickle cell disease, autoimmune disease, gender dysphoria, unexplained infertility, TS, mosaic TS, diminished ovarian reserve, medical reasons not otherwise specified.

** cancer, TS, gender dysphoria, galactosemia, impending ovarian failure, benign ovarian, autoimmune disease, benign haematological, neurological disease.

***cancer, beta thalasaemia, aplastic anaemia, paroxysmal nocturnal haemoglobinuria, gender dysphoria, TS, panhypopituitarism, NMDA autoimmune encephalitis, multiple sclerosis, benign dermoid cyst.

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17 years with those aged 18 to 21 years (43). They reported that younger participants required higher doses of gonadotropins [median 2325IU FSH (range 0-3375) versus 2038IU (range 525-5850)] and froze fewer oocytes [median 11 (range 1-24) versus 13 (2-27)]. These differences were not, however, statistically significant. A retrospective study demonstrated that younger cohorts were also more likely to have cycles cancelled because of poor response (10% in those under 20-years, compared to 4.9%, 4.7% and 7.4% in the 20-29 year, 30-34 years and ≥35 year age groups respectively) (41). For those that proceeded, however, it was concluded that OS cycles in adolescent women were similar with regard to stimulation characteristics and oocyte yield to those in other age groups.

3.3 Outcomes according to clinical diagnosis

The four large cohort studies provided grouped data on diagnoses, which included cancer, haemoglobinopathies, aplastic anaemia, paroxysmal nocturnal haemoglobinuria, gender dysphoria, TS, panhypopituitarism, N-methyl D-aspartate (NMDA) autoimmune encephalitis, multiple sclerosis, benign dermoid cyst, galactosemia and unspecified (30, 41–43) (Table 1). For the remaining 19 studies with 64 participants, there were 29 patients who were transgender (23–29), 15 patients with a sex chromosome disorder (10, 38, 39), 10 patients with a cancer diagnosis (31–36, 40), one patient with aplastic anaemia (34), one patient with pulmonary hypertension (37) and one patient with sickle cell disease (19).

Those with TS or TS mosaicism cryopreserved a mean of 3.4 mature oocytes (range 0-16) (10, 38–40), compared with a mean of 12.3 mature oocytes (range 1-23) in all extractable cancer diagnoses (30–36) and 14.7 mature oocytes (range 3-35) in transgender males (24–30). One study described eight patients with Sickle Cell Disease who had a median of nine oocytes cryopreserved (range 1-30) (19) and another study described one patient with pulmonary hypertension who had 14 oocytes cryopreserved (37). Across all studies, five patients were not successful in retrieving any oocytes, over a total of 17 cycles (39). All these patients were diagnosed with either TS or mosaic TS.

Four transgender (TG) males described in four different case studies had treatment with GnRHa to suppress puberty prior to fertility preservation. In three of these patients the mean duration of GnRHa use was 3 years (range 2-5), and in the other patient the duration of use was not described. One study described a 16-year-old who commenced GnRHa therapy at 14 years of age, at Tanner stage 2 but menarcheal status not described, who continued this throughout the period in which the oocytes were obtained and cryopreserved: four mature oocytes were cryopreserved (24). Another case report (26) described a 15-year-old who had been on treatment to suppress puberty since the age of 10. This patient had their GnRHa implant removed prior to OS and an aromatase inhibitor was used to maintain low oestrogen concentrations during OS. Despite this, 22 mature oocytes were cryopreserved from one OS cycle. In another study a 15 year-old, who had puberty suppressed since 12 years old, continued GnRHa throughout the stimulation and retrieval, and had 12 oocytes cryopreserved from one cycle (27). One patient in a recent case series used GnRHa to suppress puberty and successfully cryopreserved 25 oocytes after one OS cycle (29). It is not clear in this study if this patient continued with the GnRHa suppression throughout the OS and at what pubertal stage this was commenced.

The only study directly comparing two cohorts with different diagnoses compared nine adolescent transgender males who had not used GnRHa, with 39 adolescent females with a cancer diagnosis. There was no significant difference in the mean age between the two groups (16.4 vs 15.5 years, respectively, $P = 0.064$). There was no difference in the mean number of days of FSH stimulation between them, however the amount of FSH used was significantly lower and the peak oestradiol levels were significantly higher among the transgender males, compared with the females (3073 pg/ml vs 1269 pg/ml respectively $P = 0.018$). Despite this, there was no significant differences in the number of retrieved oocytes (30.6 ± 12.8 vs 22 ± 13.2, $P=0.091$), the number of mature oocytes (25.6 ± 12.9 vs 18.8 ± 11.2, $P=0.134$) and the maturity rates (81.5 ± 10.0% vs 85.4 ± 14.6%, $P=0.261$) of oocytes between the two groups respectively (30).

3.4 Outcomes according to ovarian reserve testing

Some form of ovarian reserve testing was performed prior to commencing ovarian stimulation in 19 studies, and these values were analysed where possible (Table 1), however, reporting of these results was incomplete. Out of 468 participants, anti-mullerian hormone (AMH, ng/mL) was described in 52 participants (10, 19, 25–29, 31, 34–36, 38–40, 43), follicular stimulating hormone (FSH, mIU/mL) in 33 participants (10, 19, 24–26, 29–33, 38–40) and antral follicle count (AFC) in 25 participants (10, 19, 25, 26, 31–36, 40). Median AMH was 2.9ng/mL (range 0.003-12.9), median FSH was 4.5mIU/L (range <0.1-20.5) and median AFC was 16 (range 5-35).

There were some ovarian reserve testing results that were outside of standard expected ranges. Four patients described in one study (39) had FSH < 1mIU/mL. These patients aged 14-18 years old, all had a diagnosis of TS or TS mosaicism and had a median of 9.5 (0-19) oocytes retrieved and a median of 5 (0-15) oocytes cryopreserved. One further case report demonstrated FSH < 1mIU/mL in a TG male on GnRHa for puberty suppression (24). This patient had five oocytes retrieved, of which four were cryopreserved in one OS cycle. There was only one patient described with FSH > 10mIU/mL (39). This 14-year-old with TS had FSH of 20.6mIU/mL and AMH 0.03ng/mL and no oocytes were retrieved over three cycles. A further 11 patients of varying ages with diagnoses including transgender males, cancer and TS or TS mosaicism, had AMH <1.1ng/mL. The median number of oocytes retrieved was 12.8 (0-33), and cryopreserved was 8.7 (0-21). Four patients aged 7-15 years old with either TS or TS mosaicism had AFC < 7 indicating low functional ovarian reserve (10, 19, 40). These patients had a mean number of 9.2 (range 0-19) oocytes retrieved, and 5.2 (range 0-9) oocytes cryopreserved over five cycles.

A low AFC (<7) was observed in one 14-year-old with leukaemia however 21 oocytes were retrieved, and 10 were cryopreserved over one OS cycle (40).

Outcomes for ovarian reserve testing outside of expected ranges were correlated with oocytes retrieved and cryopreserved (Table 2).

3.5 Outcomes according to stimulation protocol

All protocols except those described in five studies were random start antagonist cycles that used recombinant FSH +/- human menopausal gonadotrophin for ovarian stimulation (Table 1) (10, 19, 24, 33, 37). One study described the failure of a GnRHa trigger to produce oocytes in a prepubertal child with TS, with subsequent success with hCG trigger in a second cycle (10). All other studies with premenarchal patients or those using GnRHa for pubertal suppression had successful oocyte retrieval following an hCG trigger (24, 26, 27, 31, 39). The remaining post pubertal patients, not on treatment to suppress puberty, had a combination of hCG and GnRHa trigger (19, 23, 25, 27–30, 32, 33, 35, 36, 38, 43). A 15-year-old transgender male (26) also commenced aromatase inhibitor (letrozole) during OS to maintain low oestrogen concentrations. Medication doses varied depending on individual protocols and patient characteristics and were therefore not comparable.

Two female patients ≤ 18 years old underwent a double ovarian stimulation (DuoStim) protocol for fertility preservation in one study (34). In these, a 17 year old with aplastic anaemia had nine oocytes cryopreserved in the first cycle and a further 12 oocytes cryopreserved in the second cycle with a five day interval between cycles. The other, a 17 year old with myelodysplasia had one oocyte cryopreserved in the first cycle, and a further 12 oocytes cryopreserved in the second cycle after a seven day interval. Treatment as planned prior to OS was not delayed and there were no reports of OHSS in either of these patients. In four studies (10, 29, 38, 39), more than one cycle was completed, which were either in transgender patients (2) or those who had a sex chromosome disorder (8).

Both transabdominal and transvaginal ultrasound were utilised throughout the studies to monitor follicular growth and maturation during stimulation. In one seven year old patient, transabdominal oocyte retrieval was performed, in which six oocytes were successfully retrieved (10). In 133 patients transvaginal retrieval was described (19, 26, 36, 38–40, 43), and in the remaining 334 participants retrieval method was not specified (23–25, 37, 41, 42).

3.6 Adverse outcomes

In all combined studies, complications were rare (<1%). The largest study was reported by Hipp et al. (41) which included 449 patients (of whom 306 were ≤ 18 years). Data on adverse outcomes was reported as group data, (comparing ages <20 years old with 20-29 years, 30-34 year and ≥ 35 years) and a more detailed sub-analysis in those ≤18 years was not available. They reported that there was a significantly increased risk of OHSS in those younger than 20 years of age (0.9%) compared to older women (0.4%). Other complications were also rare (<1%). In this study, in women <20 years, three women (0.67%) were either hospitalized or developed an infection. A further two patients described in two different studies (19, 29) experienced mild to moderate OHSS with one of these patients requiring three days of hospital admission for supportive treatment. In both these patients hCG was used to trigger oocyte maturation.

TABLE 2 Description of studies with ovarian reserve testing results outside of expected range.

Study	Age and diagnosis	AMH (ng/mL)	FSH (mIU/mL)	AFC	Oocytes retrieved, cryopreserved
Rothenberg et al, USA (24)	16, TG male	–	0.89	–	5,4
Barrett et al, USA (29)	13-15, TG male 16-18, TG male 16-18, TG male	0.73 0.44 0.59	–	–	5,5/15,8 (2 cycles) 9,8 33,21
Reichman et al, USA (31)	13, myelodysplastic syndrome	0.95	5.0	9	20,18
Garg et al, USA (35)	14, Hodgkin's Lymphoma	0.4	–	11	13,11
Lavery et al, UK (19)	15, Sickle Cell Anaemia	–	4.8	6	5,4
Oktay et al, USA (38)	14, TS mosaicism	0.9	5.3	–	11,8
Azem et al, Israel (10)	7, TS mosaicism	1.1	5.2	5/3 (2 cycles)	0,0/6,6 (2 cycles)
Martel et al, USA (39)	14, TS 14, TS 15, TS mosaicism 15, TS 16, TS mosaicism 18, TS mosaicism	<0.16 0.03 <0.003 Unknown 1.63 Unknown	0.4 20.6 1.8 0.2 0.5 <0.1	–	4,2 0,0 0,0 15,15 19,8 0,0
Oktay et al, USA (40)	13, TS mosaicism 13, TS 14, Leukemia	1.59 0.76 0.8	5.7 5.6 7.8	6 6 5	19,9 16,7 21,10

The mental burden due to treatment-related dysphoria in transgender males undergoing OS was also described in a 16-year-old transgender male who had vaginal bleeding for 7 days after oocyte retrieval and breast development. The patient reported depressed mood and brief passive suicidal thoughts in response to these symptoms (24), which regressed within 3 months.

No study commented on delays to cancer treatment or other therapy as a result of OS.

3.7 Pregnancy using cryopreserved oocytes

Only one study reported a pregnancy resulting in a live birth after long-term cryopreservation of oocytes, from a 17 year old female requiring gonadotoxic treatment for Pulmonary Hypertension (37). The oocytes were warmed after 5 years of storage and 2 embryos were transferred into a surrogate, due to the maternal medical condition, resulting in a healthy baby boy, delivered at term weighing 3,600g. No other patients have been reported to have utilised their frozen oocytes to create a pregnancy.

4 Discussion

Fertility preservation is very important to those requiring gonadotoxic treatments or those with medical conditions that impact future fertility, and as such is a rapidly expanding field. With advancements in cryopreservation methods over the past decade in the adult population, success rates with oocyte cryopreservation have improved significantly (44) but this approach remains poorly studied and understood in the paediatric and adolescent population.

This review included 468 participants who underwent a total of 488 OS cycles, with successful mature oocyte cryopreservation in all but 18 of these cycles (96.3%). An additional 53 cycles were cancelled for poor response (9.8%) however cancellation rates should be interpreted with caution due to the retrospective nature of the studies. This systematic review therefore demonstrates that OS and oocyte cryopreservation is achievable in the young although numbers remain small and long-term outcomes unknown. Of note, three studies broadly comparing the adolescent population with the adult population (41–43) reassuringly displayed no significant different number of oocyte cryopreserved between the different age cohorts. Outside of the larger cohort studies in this review, there was a trend to higher numbers of cryopreserved oocytes in the older age ranges [median 4.5 (range 0-6) in ≤ 12 years old, median 9.5 (range 0-22) in 13-15 year-olds, median 14 (range 0-35) in 16-18 year-olds]. The number of patients are however small.

Until recently, OS has only been described in post pubertal patients. There was only one patient in this study who was prepubertal and was successful in cryopreserving six oocytes. Another premenarchal patient with TS had a low yield of two mature oocytes. However, the third premenarchal patient, with a diagnosis of myelodysplastic syndrome, had 18 mature oocytes successfully cryopreserved in one cycle. This does challenge the traditional thinking that oocyte collection can only be considered in those who are physically and emotionally mature. But questions around the number and quality of such oocytes required to achieve parenthood remain unanswered. TS or TS mosaicism had a much lower rate of successful OS and oocyte retrieval with a mean of 3.4 mature oocytes frozen (range 0-16), compared with 12.3 (range 1-23) in all extractable cancer diagnoses and 14.7 (range 3-35) in transgender males. Patients with TS are known to have a greatly increased rate of oocyte depletion resulting in low or absent ovarian reserve (2, 10) and even where follicles are present, many of these follicles may show abnormalities that are likely to limit their potential to support fertility (45).

The data from this review show that the patients who had the greatest number of oocytes frozen per cycle were the transgender patients (25, 27, 29) and this included the four transgender males who had commenced GnRHa to suppress puberty prior to fertility preservation. All four of these patients were successful in cryopreserving mature oocytes although the number of oocytes varied from 4 to 25 and their pubertal and menarchal status were not always clear or available. Although these initial data are promising, more research is required to assess the impact of initiation of GnRHa for suppression of puberty, as well as ongoing gender-affirming hormone treatment, prior to and during OS cycles.

Regardless of diagnoses, there is a paucity of data regarding utility and pregnancy outcomes from oocyte cryopreservation in young patients and there is evidence that the prepubertal ovary contains significant numbers of follicles with abnormal morphology that seem to be lost during adolescence (17). Additionally, higher rates of fetal aneuploidy have been, higher rates of fetal aneuploidy have been described in adolescent pregnancy, when compared with women in their twenties (46). Therefore, the future ability to attain a viable pregnancy and live birth is uncertain, especially in the prepubertal cohort. Only a single case report of a 17 year old female who cryopreserved oocytes resulting in a successful pregnancy and livebirth (37) is described in the literature. Future studies should focus on prospective follow-up on long term reproductive outcomes, as well as assessing additional risk or long-term implications of stimulation of an immature Hypothalamic Pituitary Ovarian (HPO) axis.

The use of standard markers of ovarian reserve such as AMH and AFC in predicting response to OS in adolescents remains unclear (47). There is a discrepancy between unfavourable test results of ovarian reserve and the associated number of oocytes cryopreserved in some cases in this study. This is thought to be multifactorial in origin and could reflect differences in the stages of ovarian development at extremes of youth (19, 35). Reassuringly there were no examples of patients with normal ovarian reserve as indicated from testing, who then responded poorly to OS. In addition to markers of ovarian reserve, standardized monitoring and stimulation protocols in the paediatric and adolescent population are not well established and the variation in stimulation protocols amongst studies created challenges when comparing data. The only study in this review to use a DuoStim protocol (34) showed promising results with an increased number of oocytes retrieved in the second cycle, increasing the number of oocytes stored. Larger studies are required to establish appropriate

assessment of ovarian reserve as well as designing optimum OS protocols in this population.

As the transvaginal ultrasound approach is often considered unacceptable in a young cohort, transabdominal ultrasound of the ovaries was frequently utilised for monitoring ovarian response to OS in the studies included in this review. Additionally, one study has described successful transabdominal oocyte retrieval in a prepubertal girl (10). The transabdominal approach of monitoring and retrieval is more technically challenging and superior visualization is generally achieved with a transvaginal probe in mature adults. It is therefore an important area of future research to assess the level of accuracy when monitoring ovarian reserve and successfully retrieving oocytes via a transabdominal approach.

It is essential to minimise the risk of harm from OS in the paediatric and adolescent population and consider the risks and benefits of this approach compared to ovarian tissue preservation (Table 3). This review demonstrates that the risk of OHSS exists, but appears to be no greater than in the adult, where the incidence of moderate and severe OHSS have been estimated to be 3-6% and 0.1-2% respectively (51). The absence of immediate embryo transfer contributes to this (52). Despite this, in the pre or peripubertal population with immature HPO axis, or the transgender populations where HCG trigger is often preferred, there is the potential for a higher risk of OHSS (53). In both cases of OHSS described in this review, where data about stimulation protocol were available, hCG was used as trigger (19, 29). Although the risks of OS and oocyte retrieval are not considered to be higher in those with TS, risk of death during pregnancy is increased by as much as 100-fold (54). Therefore, any patient who is deemed to have increased medical risk associated with carrying a pregnancy should be counselled about the option of surrogacy (55). Other medical conditions, such a sickle cell disease or cancer have a known predisposition to thrombosis and vasoocclusive events (56) underlying comorbidities which may affect safety during ovarian stimulation and ovarian response must be considered when assessing the value and mitigating risks of OS. Furthermore, it is known that the process of OS may be physically and emotionally demanding in an adult population, however the psychological impact in a young population is unknown. The risk of mental burden due to dysphoric triggers in transgender males undergoing OS is an important consideration as the process increases endogenous oestrogen production, may involve discontinuing or reducing the dose of testosterone or other gender affirming hormonal treatments, and the resumption of menses before beginning the process (57, 58).

In those utilising OS for oocyte cryopreservation prior to cancer related therapies, current evidence does not suggest differences in survival and recurrence of cancer rates in adult patients who underwent OS prior to gonadotoxic cancer treatments compared with those who did not (59, 60) although this has yet to be studied in those 18 and younger. Furthermore OS is not considered a viable option in those in poor general condition who need to commence cancer treatment straight away, resulting in reportingc bias.

There were limitations in evaluating this review that may have impacted the analysis of outcomes. The description of ovarian reserve markers as well as baseline patient characteristics including BMI, Tanner stage and menstrual history was described in varying detail and often lacking amongst the studies. This could affect the comparison between patients and as such, results in this study should be interpreted with caution. Furthermore, discrepancies in monitoring and stimulation protocols amongst studies could impact the ability to compare overall outcomes.

The purpose in each study varied, with some studies comparing different diagnoses in their analysis and others comparing differing ages. Other studies reported broad outcomes for cohorts that included all ages from childhood to adulthood and encompassed

TABLE 3 Pros and Cons of ovarian tissue cryopreservation compared to Oocyte Cryopreservation in those ≤ 18 years.

Ovarian tissue cryopreservation	Oocyte cryopreservation
Two reported pregnancies from prepubertal tissue, innovative procedure, now transitioned into standard practice (12, 13)	One pregnancy from post-pubertal oocyte collection. Consider experimental in prepubertal patients, innovative in post-pubertal patients under 18 years (37)
No delays to cancer therapy	Two-week delay to treatment, cannot be offered to those who require urgent cancer treatment
Can be done at any age	The youngest case report is 7 years of age (10)
No lengthy monitoring required for tissue harvest	Requires hormone treatment, monitoring with blood tests and scans which may cause morbidity in gender diverse and other populations
Requires careful selection to minimise morbidity	Requires careful selection to minimise morbidity
Minimally invasive surgery, low-risk procedure with careful patient selection (48)	Risks ovarian hyperstimulation syndrome <1%, and other complications<1%
May be undertaken as interval procedure after start of gonadotoxic therapy	Cannot be undertaken for at least 6 months after gonadotoxic therapy due to mutagenic risk (49)
Provides very high follicle density numbers: adult data suggests 25% chance of livebirth, success rates in the young are unknown (50)	Provides a finite number of oocytes: adult data suggests cumulative live births per patient 33.9-35.2% for women under 35 years, but success rates in the young are unknown (15)
Autotransplantation in gender diverse populations may not be tolerable to them. Carries a risk of malignant reseeding in some populations (3, 20)	Does not require auto-transplantation of tissue
Oocytes with abnormal morphology likely to undergo atresia	May theoretically increase yield of oocytes with abnormal morphology (17)

a variety of diagnoses. Many of the larger studies in this review were not able to provide a breakdown of age in their description of results. The range of diagnoses and ages throughout the studies in this review may have significant impacts on the likelihood of success of COS making the results not necessarily applicable to alternate populations.

5 Conclusion

OS and oocyte cryopreservation is novel in the paediatric and adolescent population, but it offers hope to younger people and more diverse patient populations for the possibility of future biological parenthood. While it is considered standard practice in adults, long term outcomes are largely unknown in the young and the procedure should be considered experimental in prepubertal and premenarchal patients.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

Conception and design: MS, MP, KM, DL, RA, KS, DG, YJ. Data Acquisition, Analysis: MS, KM. Data interpretation: MS, MP, KM, DL, RA, KS, DG, YJ. Critically reviewing drafts: MS, MP, KM, DL, RA, KS, DG, YJ. Critical review and approval of final draft: MS, MP, KM, DL, RA, KS, DG, YJ. Resources and supervision: MS, MP, YJ. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1146476/full#supplementary-material

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Appendix 1

TABLE 1 Checklist for assessing the quality of quantitative studies.

Criteria		YES (2)	PARTIAL (1)	NO (0)	N/A
1	Question / objective sufficiently described?				
2	Study design evident and appropriate?				
3	Method of subject/ comparison group selection or source of information/input variables described and appropriate?				
4	Subject (and comparison group, if applicable) characteristics sufficiently described?				
5	If interventional and random allocation was possible, was it described?				
6	If interventional and blinding of investigators was possible, was it reported?				
7	If interventional and blinding of subjects was possible, was it reported?				
8	Outcome and (if applicable) exposure measure(s) well defined and robust to measurement / misclassification bias? Means of assessment reported?				
9	Sample size appropriate?				
10	Analytic methods described/justified and appropriate?				
11	Some estimate of variance is reported for the main results?				
12	Controlled for confounding?				
13	Results reported in sufficient detail?				
14	Conclusions supported by the results?				

Appendix 2

TABLE 2 Risk of bias assessment.

Author	1. Objective	2. Design	3. Subject selection	4. Subject characteristics	5. Random allocation	6. Blinding investigators	7. Blinding subjects	8. Outcomes	9. Sample size	10. Analysis	11. Estimate of variance	12. Cofounding	13. Results	14. Conclusion	Total Score (%)
Maxwell et al. (23)	No (0)	Yes (2)	Yes (2)	Partial (1)	N/A	N/A	N/A	Yes (2)	Yes (2)	No (0)	No (0)	No (0)	Partial (1)	Yes (2)	54%
Rothenberg et al. (24)	No (0)	Partial (1)	Yes (2)	Partial (1)	N/A	N/A	N/A	Yes (2)	Yes (2)	No (0)	No (0)	No (0)	Yes (2)	No (0)	45%
Wallace et al. (25)	No (0)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	73%
Martin et al. (26)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Yes (2)	77%
Insogna et al. (27)	No (0)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Partial (1)	64%
Chen et al. (28)	No (0)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Partial (1)	64%
Barrett et al. (29)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	82%
Amir et al. (30)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	Yes (2)	Yes (2)	Yes (2)	91%

(*Continued*)

TABLE 2 Continued

Author	1. Objective	2. Design	3. Subject selection	4. Subject characteristics	5. Random allocation	6. Blinding investigators	7. Blinding subjects	8. Outcomes	9. Sample size	10. Analysis	11. Estimate of variance	12. Cofounding	13. Results	14. Conclusion	Total Score (%)
Reichman et al. (31)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Partial (1)	73%
Peddie et al. (32)	Partial (1)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	78%
Cai, H et al. (33)	No (0)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Partial (1)	68%
Tsampras et al. (34)	No (0)	Yes (2)	Yes (2)	Partial (1)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Partial (1)	64%
Garg et al. (35)	No (0)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Partial (1)	64%
Kutteh et al. (36)	No (0)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Partial (1)	64%
Kim at al (37)	Yes (2)	Yes (2)	Yes (2)	Partial (1)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Partial (1)	68%
Lavery et al. (19)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	82%
Oktay et al. (38)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Partial (1)	No (0)	No (0)	Yes (2)	Partial (1)	73%
Azem et al. (10)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	82%
Martel et al. (39)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	82%
Oktay et al. (40)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	82%
Hipp et al. (41)	Yes (2)	Yes (2)	Yes (2)	Partial (1)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	Yes (2)	No (0)	Yes (2)	Yes (2)	86%
Rodriguez-Wallberg et al. (42)	Yes (2)	Yes (2)	Yes (2)	Partial (1)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	No (0)	Yes (2)	Yes (2)	78%
Manuel, et al. (43)	Yes (2)	Yes (2)	Yes (2)	Partial (1)	N/A	N/A	N/A	Yes (2)	Yes (2)	Yes (2)	No (0)	Yes (2)	Yes (2)	Yes (2)	86%

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Case Report: Longitudinal follow-up and testicular sperm extraction in a patient with a pathogenic *NR5A1* (SF-1) frameshift variant: p.(Phe70Ser*fs**5)

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Background: Steroidogenic factor 1 (SF-1), encoded by the nuclear receptor subfamily 5 group A member 1 (*NR5A1*) gene, is a transcriptional factor crucial for adrenal and gonadal organogenesis. Pathogenic variants of *NR5A1* are responsible for a wide spectrum of phenotypes with autosomal dominant inheritance including disorders of sex development and oligospermia–azoospermia in 46,XY adults. Preservation of fertility remains challenging in these patients.

Objective: The aim was to offer fertility preservation at the end of puberty in an *NR5A1* mutated patient.

Case report: The patient was born of non-consanguineous parents, with a disorder of sex development, a small genital bud, perineal hypospadias, and gonads in the left labioscrotal fold and the right inguinal region. Neither uterus nor vagina was detected. The karyotype was 46,XY. Anti-Müllerian hormone (AMH) and testosterone levels were low, indicating testicular dysgenesis. The child was raised as a boy. At 9 years old, he presented with precocious puberty treated by triptorelin. At puberty, follicle-stimulating hormone (FSH), luteinising hormone (LH), and testosterone levels increased, whereas AMH, inhibin B, and

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testicular volume were low, suggesting an impaired Sertoli cell function and a partially preserved Leydig cell function. A genetic study performed at almost 15 years old identified the new frameshift variant NM_004959.5: c.207del p.(Phe70Ser*fs**5) at a heterozygous state. He was thus addressed for fertility preservation. No sperm cells could be retrieved from three semen collections between the ages of 16 years 4 months and 16 years 10 months. A conventional bilateral testicular biopsy and testicular sperm extraction were performed at 17 years 10 months of age, but no sperm cells were found. Histological analysis revealed an aspect of mosaicism with seminiferous tubules that were either atrophic, with Sertoli cells only, or presenting an arrest of spermatogenesis at the spermatocyte stage.

Conclusion: We report a case with a new *NR5A1* variant. The fertility preservation protocol proposed at the end of puberty did not allow any sperm retrieval for future parenthood.

KEYWORDS

testicular sperm extraction, gonadal dysgenesis, spermatogenesis, male infertility, congenital, disorder of sex development, hypospadias, azoospermia

1 Introduction

Steroidogenic factor 1 (SF-1) is a transcription factor crucial for adrenal and testis organogenesis as well as steroidogenesis regulation with a dose-dependent effect (1–3).

SF-1 protein is encoded by the nuclear receptor subfamily 5 group A member 1 (*NR5A1*) gene located in chromosome 9 and composed of seven exons. SF-1 protein is characterised by a DNA-binding domain (DBD) in the amino-terminal region and by a ligand-binding domain (LBD) in the carboxy-terminal region separated by a hinge region, which can host post-translational changes (2, 4, 5).

To date, more than 180 putative pathogenic variants have been reported in *NR5A1* coding regions and splice sites, spanning the whole gene and including missense variants (58% of variants), frameshift variants (18.6%), non-sense variants (12.3%), and splice variants (3.3%) in a heterozygous and isolated state in almost all cases. Variants were *de novo* in almost half of the cases and autosomal dominant inheritance in the others (4).

Pathogenic variants in *NR5A1* are responsible for almost 20% of 46,XY differences or disorders of sex development (DSDs) (4). 46, XY DSD related to mutated *NR5A1* is characterised by a wide spectrum of phenotypes, from male to female external genitalia and including partial or complete dysgenesis, genital ambiguity, micropenis, hypospadias, cryptorchidism, and asplenia with no clear genotype–phenotype correlation. Adrenal insufficiency is rarely associated with the picture (4, 6).

Severe oligospermia or azoospermia can be found in 46,XY patients carrying *NR5A1* pathogenic variants; sometimes, these are the only symptoms (7, 8). Fertility care in 46,XY patients presenting azoospermia and carrying *NR5A1* pathogenic variants has rarely been studied. Among these patients, only four cases who underwent testicular sperm extraction (TESE) have been reported, with inconsistent results (Table 1), underlining that preservation of fertility in such cases is challenging.

Here, we report a longitudinal follow-up from birth to adulthood of a patient carrying a novel frameshift variant of *NR5A1*. We focused our follow-up on the physical and hormonal evaluations particularly during the puberty period, and also on testicular histology and semen collections.

2 Case report

The patient was born to non-consanguineous parents at 37 weeks of amenorrhea with a tetralogy of Fallot and a DSD. Longitudinal morphological and laboratory data are summarised in Table 2.

At birth, the patient had a small genital bud (6 mm long) with perineal hypospadias. A gonad of 9 × 6 mm was palpated in the left labioscrotal fold, and the right gonad was in the high inguinal region. The genitography did not show any uterus or vagina. Karyotype and fluorescence *in situ* hybridisation showed a normal 46,XY formula. Anti-Müllerian hormone (AMH) levels were low on the first day of life (64 pmol/L) and at the

Abbreviations: ACMG, American College of Medical Genetics and genomics; AMH, anti-Müllerian hormone; DBD, DNA-binding domain; DSD, disorder of sex development; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; LH, luteinising hormone; LBD, ligand-binding domain; NMD, non-sense-mediated mRNA decay; NR5A1, nuclear receptor subfamily 5 group A member 1; SF-1, steroidogenic factor 1; TESE, testicular sperm extraction.

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TABLE 1 Fertility preservation in mutated *NR5A1* 46,XY men presenting azoospermia.

Patient	*NR5A1* variant (NM_004959.5, GRCh37/hg19)	Clinical description at birth	Hormonal description (closest to TESE)	Gonadal description	TESE procedure and results (age)	Reference
1	c.118A>C p.(Thr40Pro) Heterozygous	Ambiguous genitalia, palpable gonads in labioscrotal folds, no Müllerian remnant at birth	FSH ↗ LH ↗ Testosterone subN Inhibin B ↘	Hypoplastic testis, neonatal testicular appearance on histology at birth Low TV (L and R: 4-6 ml) at 15.8 y-o	TESE: no sperm cells (18 y-o)	(9)
2	c.39C>A p.(Cys13*) Heterozygous	Bilateral cryptorchidism treated by orchidopexy	FSH ↗ LH N Testosterone N	Low TV (L: 6.6 ml, R: 10.1 ml) at adult age	Micro-TESE after 3 months of vitamin E and clomiphene citrate: few sperm cells, but the number was not specified (20 y-o)	(10)
3	c.730A>G p.(Ile244Val) Heterozygous	Not available	FSH N LH N Testosterone N	Normal TV (L: 13.4 ml, R: 14.1 ml) at adult age	Micro-TESE after 3 months of vitamin E and clomiphene citrate: few sperm cells, but the number was not specified (35 y-o)	(10)
4	c.244+1G>A Heterozygous	Not available	FSH ↗ LH ↗ Testosterone subN	Low TV (L: 4.1 ml, R: 5.4 ml), normal spermatogenic function on histology at adult age	Micro-TESE: sufficient sperm cells for ICSI (31 y-o)	(10)

FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; L, left; LH, luteinising hormone; N, normal; R, right; subN, subnormal; TESE, testicular sperm extraction; TV, testicular volume; y-o, years old; ↘ or ↗, decreased or increased, respectively.

minipuberty (92.5 pmol/L). Testosterone level was also low at the 12th hour of life (0.90 nmol/L) and was stimulated to 10.85 nmol/L after a human chorionic gonadotropin (hCG) test (seven injections of 1,500 IU every 2 days). On the 14th day of life, follicle-stimulating hormone (FSH) and luteinising hormone (LH) levels were high (9.5 and 9.2 IU/L, respectively).

The patient received four injections of heptylate testosterone (two doses of 20 mg and then two doses of 25 mg, 15 days apart) and was declared male at 3 months of age. Several surgical treatments were performed, first for his hypospadias at 1 year of age, then for the undescended testis at 2 years 6 months, and finally for the correction of the penis curvature at 9 years. At 9 years, precocious puberty was suspected due to an increase in testicular volume (TV), and a gonadotropin-releasing hormone (GnRH) test confirmed a central origin. Magnetic resonance imaging of the hypothalamo-pituitary region was normal. GnRH analog treatment (triptorelin: one injection every 4 weeks and then every 3 weeks due to insufficient effectiveness) was introduced from 9 years 10 months to 11 years 4 months of age. From the age of 13 years 6 months to 14 years 10 months, testosterone enanthate (50 to 125 mg, one injection every 3 weeks) was undertaken since LH was high and to improve penis size prognosis but was interrupted because of its poor effectiveness. At 14 years old, a varicocele on the left side was observed and highlighted by testicular echography.

At 16 years 4 months of age, he was addressed for fertility preservation. TV was diminished (6 ml on both sides), and AMH and inhibin B levels were low. Three semen collections performed according to the 2010 World Health Organization criteria (15) between the ages of 16 years 4 months and 16 years 10 months retrieved no sperm cells. The varicocele on the left side was treated by embolisation at the age of 17 years 2 months. The patient was eligible for a testicular biopsy with testicular sperm extraction (conventional TESE) since no sperm cells were found in at least two sperm samples 3 months apart. TESE was practiced according to the procedure described previously (16) when the patient was 17 years 10 months old. Only one sperm cell was found on a right testis fraction, but this was insufficient for cryopreservation.

The histological analysis of biopsy fragments revealed a severely impaired spermatogenesis with an aspect of histological mosaicism using Johnsen score (17): the seminiferous tubules, of overall reduced diameter and lined by a thick basal membrane, were either atrophic, with Sertoli cells only, or with spermatogenesis arrest at the spermatocyte stage. The interstitial tissue was fibro-edematous with hyperplastic Leydig cells. No signs of malignancy or dysplasia were noticed (Figure 1).

At 14 years 10 months of age, after his parents provide signed informed written consent for genetic testing, a molecular analysis of *NR5A1* gene (Sanger sequencing on DNA extracted from whole blood) revealed the unreported heterozygous frameshift variant NM_004959.5: c.207del p.(Phe70Ser*fs**5) (GRCh37/hg19) (Figure 2). His parents and his sibling were not sequenced for *NR5A1*. According to the American College of Medical Genetics and Genomics (ACMG) criteria (18), this variant is classified as pathogenic. This variant had never been reported in Gnomad_v2, ClinVar, and dbSNP databases or in any literature to date. It is in the third exon of *NR5A1* gene encoding the DBD of the SF-1 protein. Since it induces a frameshift with the manifestation of a premature stop codon, it should lead to either an inactive truncated protein (truncated DBD, absence of the hinge region and the LBD) or the absence of protein by the non-sense-mediated mRNA decay

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(NMD) mechanism (19). The coding regions of the androgen receptor (*AR*) gene were also studied; no pathogenic variant was found.

Otherwise, the patient had some periods of overweight during childhood and early adulthood because of a lack of physical activity and overeating. The patient never presented with an adrenal crisis and the exploration of the hypothalamic–pituitary–adrenal axis was normal (cortisol and adrenocorticotropic hormone (ACTH)).

3 Discussion

We describe a 46,XY male patient carrying a new *NR5A1* pathogenic variant with medical monitoring from birth to adulthood, at the beginning of which a testicular biopsy with TESE was performed.

The novel variant NM_004959.5: c.207del p.(Phe70Ser*fs**5) found here was classified as pathogenic according to the ACMG criteria. Certain frameshift variants inducing a premature stop codon in the same position as those observed herein have been reported to lead to a protein with the same missing parts (truncated DBD, absence of the hinge region, and the LBD) if one is produced. The variant NM_004959.5: c.18del p.(Asp6Glu*fs**69) was found in a 46,XY patient born with ambiguous external genitalia and raised as female. At 27 years old, she had clitorimegaly, a blind-ended vagina with no uterus, severely hypoplastic inguinal testis, primary hypogonadism with high gonadotropin levels and low baseline testosterone level non-responsive to hCG stimulation, and no adrenal dysfunction (20). Another variant, NM_004959.5: c.70del p.(His24Thr*fs**51), was found in a 46,XY patient with ambiguous external genitalia at birth. The patient presented with an absence of Müllerian ducts and no palpated gonads and was initially raised as female until 18 years old. The patient had high gonadotropin levels, normal testosterone concentration, and normal adrenal function at 18 years of age, and the patient's testes were considered dysgenetics at 19 years old (21). The last variant, NM_004959.5: c.151del p.(Glu51Arg*fs**24), was found in a 46,XY adolescent patient raised as female and who presented with clitorimegaly, primary amenorrhea, and inguinal testes (22). The p.(Asp6Glu*fs**69) and p.(His24Thr*fs**51) variants were explored by functional studies that showed a reduction in the transactivation capacity of SF-1 on the promoters of some genes coding for steroidogenic enzymes. Nevertheless, Western blotting performed on transfected cells could not detect any protein, due to either NMD or the non-ability of the technique to detect small peptides (20, 21). The p.(Phe70Ser*fs**5) variant identified herein should have similar consequences on the transactivation capacity of SF-1 than the p.(Asp6Glu*fs**69) and p.(His24Thr*fs**51) variants.

The clinical and hormonal data recorded in the present patient could be integrated into the wide spectrum of phenotypes of mutated *NR5A1* patients (reviewed in (4, 23)).

At the patient's birth, AMH levels were low for a 46,XY newborn but too high for a 46,XX newborn, and total testosterone level was subnormal but increased properly after hCG stimulation in early childhood. These two parameters indicated the presence of dysgenetic testicular tissue with an

TABLE 2 Birth and follow-up morphological and laboratory data in a patient with a pathogenic *NR5A1* (SF-1) frameshift variant.

Age	Ongoing treatment	Height, weight, BMI	Penis length (mm)	Testicular volume	FSH (IU/L)	LH (IU/L)	Total testosterone (nmol/L)	Inhibin B (ng/L)	AMH (pmol/L)	Bioavailable testosterone (nmol/L)	Other examinations
H12	/	46 cm, 2.320 kg	6	9 × 6 mm (L) Not palpated (R)	/	/	0.90 ↘	/	64.0 ↘	/	/
D2	/	/	/	/		/	<0.10 (N)	/	/	/	/
D14	/	/	/	/	9.5 ↗	9.2 ↗	3.02 (subN)	/	/	/	/
D27	After 7 injections of hCG	/	16	/	/	/	10.85 (with parallel increase in dihydrotestosterone) (N)	/	92.5 ↘	/	/
1y	After 11 injections of testosterone	/	32	/	/	/	/	/	/	/	Tanner P2
9y	/	/	/	35 × 18 mm (L and R)	/	/	9.00 (↗ for age)	<30 ↘	18.7 (↘)	/	Bone age: 11y6m
9y10m	Triptorelin	BMI at 97*e* percentile	45	/	/	/	/	/	/	/	/
11y4m	Triptorelin		/	/	/	/	/	/	/	/	Bone age: 13y

(Continued)

TABLE 2 Continued

Age	Ongoing treatment	Height, weight, BMI	Penis length (mm)	Testicular volume	FSH (IU/L)	LH (IU/L)	Total testosterone (nmol/L)	Inhibin B (ng/L)	AMH (pmol/L)	Bioavailable testosterone (nmol/L)	Other examinations
12y7m	/		50	33 × 16 mm (L) 32 × 15 mm (R)	22.7 ↗	10.6 ↗	/	6 ↘	1.3 ↘	/	Tanner P4
13y6m	/	160.5 cm, 60.5 kg BMI: 23.5 (>97e percentile)	55	25 × 20 mm (L) 28 × 20 mm (R)	36 ↗	18.5 ↗	13.63 N	/	/	/	Tanner P4 Bone age: 13y6m
14y10m	Testosterone	163 cm	55-60	8 ml (L) 6 ml (R)	/	/	/	< 5 ↘	0.8 ↘	/	Bone age: 15y–16y
15y1m	/	/	/	/	29.5 ↗	25.0 ↗	9.46 ↘	/	/	/	/
16y4m	/	/	/	6 ml (L) 6 ml (R)	33.2 ↗	19.8 ↗	6.50 ↘	9.0 ↘	2.6 ↘	2.10 (subN)	SBP: 10 nmol/L
16y8m	/	165 cm, 65 kg BMI: 23.9 (around 90e percentile)	/	/	32.8 ↗	20.1 ↗	9.40 ↘	6.0 ↘	2.5 ↘	2.71 (subN)	SBP: 16 nmol/L
16y10m	/	/	/	/	29.4 ↗	19.0 ↗	13.06 N	7.0 ↘	2.5 ↘	4.38 (N)	SBP: 17 nmol/L
20y	/	172 cm (for a genetic target at 174 cm), 82 kg BMI = 27.7	/	/	/	/	/	/	/	/	/

AMH, anti-Müllerian hormone; D, day; FSH, follicle-stimulating hormone; H, hour; hCG, human chorionic gonadotropin; L, left; LH, luteinising hormone; m, month; N, normal; R, right; SBP, sex-binding protein; subN, subnormal; y, year; BMI, body mass index; ↘ or ↗, decreased or increased, respectively, according to reference range for age and sex when it exists or personal interpretation (in parentheses) when it does not.

Plasma FSH and LH were assessed by radioimmunoassay (newborn data) or by an automated chemiluminescence immunometric assay on Architect i2000SR (Abbott, Chicago, IL, USA). Reference ranges for FSH: 0.05 to 1 IU/L in prepubescent boys between 10 and 30 months, 1.1 to 7.2 IU/L in men with normal testicular function. Reference ranges for LH: 0.02 to 0.80 IU/L in prepubescent boys between 10 and 30 months, 1.3 to 5.8 IU/L in men with normal testicular function.

Plasma total testosterone was assessed by in-house radioimmunoassay after solvent extraction and chromatography or by in-house liquid chromatography coupled with tandem mass spectrometry after extraction. Reference ranges for boys: 9.36 ± 5.31 nmol/L at D1, 0.97 ± 0.38 nmol/L at D5, 5.37 ± 2.64 nmol/L between D11 and D15, 8.68 ± 2.77 nmol/L between 1 and 3 months, 0.28 ± 0.01 nmol/L (mean ± standard deviation) in prepubescent boys between 1 and 10 years old, 10.40 to 26.00 nmol/L in young men.

Serum inhibin B was assessed by enzyme immunoassay using the Inhibin B Gen II ELISA kit (Beckman Coulter, Brea, CA, USA). Reference range: 35 to 167 ng/L in boys between 6 and 10 years old, 74 to 470 ng/L in boys between 12 and 17 years old, 92 to 316 ng/L in normozoospermic men (11, 12).

Serum AMH was assessed by enzyme immunoassay (newborn data) or by an automated electrochemiluminescence assay on Cobas e601 (Roche Diagnostics, Basel, Switzerland). Reference ranges for boys: 395 to 2,321 pmol/L between D13 and D20, 505 to 3,213 pmol/L between 2.8 and 5.1 months, 705 to 4,280 pmol/L between 8.5 and 9.8 months, 441 to 2,352 pmol/L at 4 years old, 16.4 to 90.3 pmol/L in men with normal spermatogenesis (13, 14).

Plasma bioavailable testosterone was assessed by in-house radioimmunoassay after solvent extraction and chromatography. Reference range: 2.25 to 10.70 nmol/L in men between 20 and 40 years old.

SBP was assessed by radioimmunoassay with the SHBG RIACT Cisbio Kit (Cisbio Bioassays, Codolet, France). Reference range: 17 to 45 nmol/L in men.

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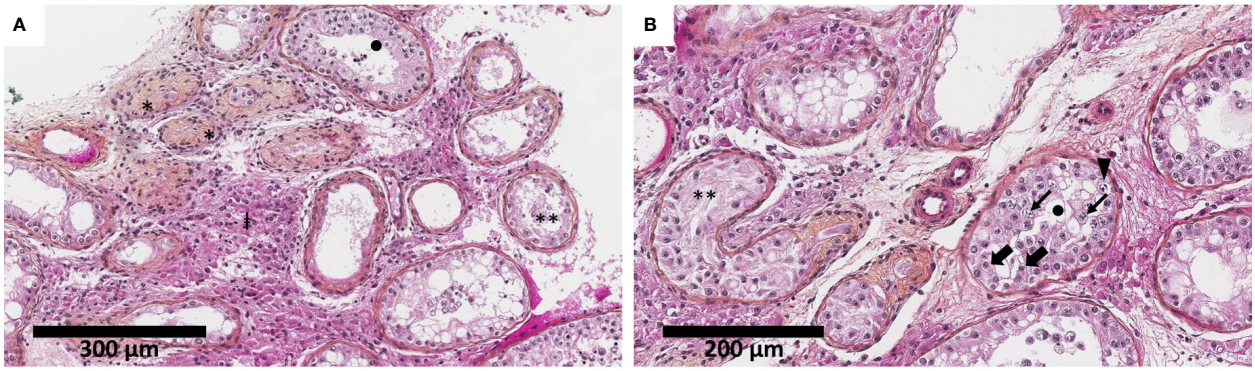

FIGURE 1
Testicular biopsy shows severely impaired spermatogenesis with an aspect of histological mosaicism. The seminiferous tubules were of overall reduced diameter (decreased by approximately 30%–50% compared to physiological adult pubertal seminiferous tubules of 150–250 µm in diameter) and lined by a thickened basal membrane. The seminiferous tubules were either atrophic (*), with Sertoli cells only (**), or presenting spermatogenesis arrest at the spermatocyte stage (•). The interstitial tissue was fibro-edematous with numerous Leydig cells (‡) (hematoxylin–eosin–saffron). Thick black arrow indicates Sertoli cells, thin black arrow indicates spermatocyte, and solid black triangle indicates spermatogonia. Biopsy fragments were fixed in alcohol, formalin, and acetic acid (AFA) and paraffin-embedded. Sections of 3 µm were stained by hematoxylin–phloxin–saffron. Slide evaluation was performed on a Leica DM2500 microscope. Two different scales: 300 µm **(A)** and 200 µm **(B)**.

impaired function more marked on Sertoli cells than on Leydig cells. However, the absence of the uterus indicated a sufficient secretion of AMH during the *in utero* life. As suggested elsewhere, foetal Sertoli cell function was thus sufficient to induce the regression of the Müllerian ducts but decreased after birth (23–25). The incompletely virilised external genitalia reported herein suggests that testosterone and dihydrotestosterone were probably insufficiently secreted during the *in utero* window of masculinisation to induce the complete development of external genitalia (26). Testosterone levels (basal or stimulated) vary greatly among 46,XY *NR5A1* mutated patients (23).

At puberty, the patient showed an insufficient increase in TV, low AMH and inhibin B levels, and elevated FSH levels that indicated a severe primary Sertoli cell injury. The normal or subnormal testosterone levels with elevated LH levels suggested a compensated primary hypofunction of Leydig cells and explained the virilisation signs observed in the patient at puberty. This description is in line with that of Mönig et al., who studied 10 *NR5A1* mutated patients during adolescence and puberty (24). Other authors also showed an impaired Sertoli cell function with a normal or subnormal Leydig cell function conserved at least until puberty (23, 25, 27).

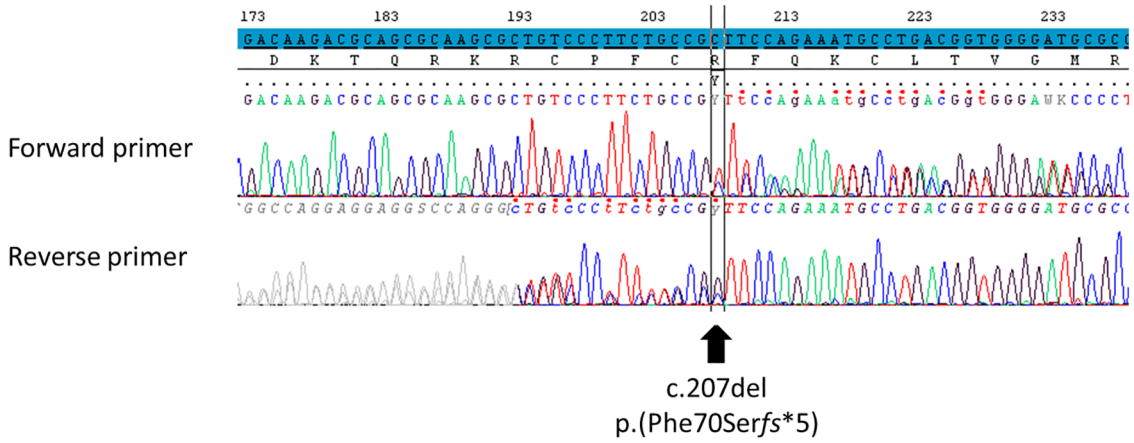

FIGURE 2
Identification of the *NR5A1* variant using Sanger sequencing. Variation was identified using reference NM_004959.5 for *NR5A1* transcript on GRCh37/hg19 human genome assembly, NP_004950.2 for SF-1 protein. The screenshot comes from SeqScape 3 software. The nucleotide reference sequence is highlighted in blue. This variant was classified as pathogenic according to the ACMG criteria (PVS1, PM1, and PM2). The polymorphism NM_004959.5: c.437G>C p.(Gly146Ala) was not found. In ACMG criteria: PM, pathogenic moderate; PVS, pathogenic very strong. "PVS1: null variant (non-sense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function is a known mechanism of disease." "PM1: located in a mutational hot spot and/or critical and well-established functional domain (e.g. active site of an enzyme) without benign variation." "PM2: absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes or ExAC.".

Interestingly, the precocious increase in TV and testosterone levels herein suggested precocious puberty confirmed by a GnRH test. The occurrence of precocious puberty was surprising because SF-1 is expressed in pituitary cells in humans and is implicated in the formation of the ventromedial hypothalamic nucleus in mice (28) and because some mutated *NR5A1* patients encountered difficulties when entering puberty spontaneously (29). However, Mönig et al. reported an early pubertal development with an early increase in testosterone levels in three out of 10 cases (24).

Amazingly, the spontaneous increase in testosterone levels and virilisation at puberty contrasted with the subnormal testosterone levels and incompletely virilised external genitalia at birth reported herein and elsewhere (23, 24). As demonstrated in mice, there is evidence in humans that testosterone synthesis during foetal life imply a coordinated action of foetal Sertoli cells and foetal Leydig cells (30). Therefore, a Sertoli cell dysfunction during foetal life may impair testosterone production by the foetal testis and lead to a lack of virilisation of the external genitalia at birth.

Furthermore, progressive degradation of testicular function with age was suggested in the literature based on several physical and hormonal observations. First, AMH was secreted during *in utero* life, but its levels were low at birth and in the neonatal period as discussed above (23–25), indicating gonadal dysgenesis. Herein, AMH levels were already low at birth. Then, a decrease in TV can occur during or after puberty (24); it was not significant herein perhaps because TV was initially too low. Finally, a progressive increase in the FSH and LH levels and a progressive decrease in testosterone and inhibin B levels with age were reported (9, 24, 25, 31). This pattern was observed herein mainly for FSH and LH levels but not inhibin B levels since the first value (at 12 years 7 months) was already too low. At 16 years 10 months, testosterone level was normal, but further degradation may not be excluded.

As expected given the low TV and low AMH and inhibin B levels, no sperm cells were retrieved in semen samples. Azoospermia was previously reported in *NR5A1* mutated patients (7), but varicocele could aggravate the spermiological phenotype in this case (32). Spermatogenesis could be improved 3 to 6 months after varicocele treatment (32).

Interestingly, some sperm cells were collected in the semen of some 46,XY mutated *NR5A1* patients (7, 8), and certain patients even fathered children naturally (25, 33–35). Among the latter, one patient had two children even though he carried an *NR5A1* pathogenic variant in a mosaic state in DNA extracted from blood leukocytes (25). One had two children at 30 and 33 years old but refused further investigations (35). One fathered five children before the age of 32 years and presented with increased FSH levels and undetectable AMH and inhibin B levels at 57 years old. However, no hormonal data were available when he was 32, and no sperm data were available for him or his boys (33). From the perspective of progressive hormonal function alteration, some authors suggested a progressive degradation of spermatogenesis with age that allows paternity in young men before spermatogenesis collapses (7, 23, 25, 31). However, this hypothesis remains to be confirmed by longitudinal sperm counts in *NR5A1* mutated 46,XY patients in whom spermatogenesis is preserved. Consequently, men carrying *NR5A1* pathogenic variants should be addressed for fertility preservation as early as possible after puberty; if mature sperm cells are retrieved, cryopreservation can thus be performed to ensure a timely medically assisted reproduction. A TESE was proposed to the patient herein when he was 17 years 10 months. Although it was performed sufficiently long enough after varicocele treatment to allow for the potential restoration of spermatogenesis, only one sperm cell was retrieved, thus preventing cryopreservation and intracytoplasmic sperm injection (ICSI) to be performed. In the literature (Table 1), one team failed to retrieve sperm cells in an 18-year-old man using TESE (9), while another retrieved sperm cells in three men (20, 31, and 35 years old) using micro-TESE after 3 months of vitamin E and clomiphene citrate for two of them (10). This discrepancy in TESE outcomes could be explained by different situations. First, based on the hypothesis of progressive spermatogenesis degradation, the age when TESE was performed may have impacted the outcomes. However, TESE retrieved sperm cells in the three older patients but failed in the youngest. Second, the TESE procedure performed: micro-TESE did not show better results for retrieving sperm cells than conventional TESE in men with non-obstructive azoospermia in a recent meta-analysis (36). Third, the wide spectrum of the disease without a clear phenotype–genotype relation likely impacts TESE outcomes. The fact that the same *NR5A1* pathogenic variant can cause different phenotypes in patients belonging to the same family (9, 33, 35, 37, 38) may suggest a possible polygenic inheritance or the intervention of additional epigenetic or environmental factors in the phenotype severity. In the case of polygenic inheritance, whole genome sequencing would be of great interest to find another mutated gene and to understand the spectrum of *NR5A1*-related diseases. Finally, features correlated with spermatogenesis function (39–41) like hormonal markers (FSH, LH, testosterone, and inhibin B), TV, and history of cryptorchidism are likely to affect TESE outcomes here: one patient in whom TESE retrieved sperm cells had a normal hormonal profile and normal TV. Unfortunately, we did not have access to the clinical description at birth or the follow-up of inhibin B levels since adolescence in all patients who underwent TESE to be able to suggest a relationship between the severity of the DSD and the TESE outcomes (9, 10).

The hormonal anomalies, sperm sampling, and TESE outcomes herein were consistent with the results of the pathological analysis of the biopsied testicular fragments. The pathological aspect observed herein was also consistent with the wide spectrum of testicular biopsy descriptions found elsewhere in 46,XY *NR5A1* mutated adults (8, 23, 42). The possible degradation of testicular function in terms of hormonal and sperm parameters with aging discussed above might parallel a progressive degradation of testis structure observable on the testicular biopsy, as suggested by Camats et al. (21). Nevertheless, if testicular biopsy finds germ cells and functional seminiferous tubules, future techniques of fertility preservation, such as the emerging *in vitro* spermatogenesis technology (43, 44), would be of great interest in *NR5A1* mutated patients with azoospermia.

Although overweight in *NR5A1* mutated patients has already been described (20, 45, 46), this feature was not found in all patients (46). Interestingly, the homozygous deletion of *NR5A1* in an SF-1 knock-out mouse model induced obesity (47). In line with this finding, some authors suggested the intervention of SF-1 in the

development of the ventromedial hypothalamic nucleus, a central player in appetite regulation in humans (20, 46).

Finally, the presence of tetralogy of Fallot is surprising and was not reported elsewhere in association with a mutated SF-1. However, we could not exclude an additional genetic anomaly in other genes associated with DSD and/or tetralogy of Fallot, *GATA4* and *ZFPM2/FOG2* genes, for example (48, 49).

4 Conclusion

We report a case with a new *NR5A1* pathogenic variant addressed for fertility care. The physical, hormonal, and histological description of the testis could be integrated into the wide spectrum of 46,XY DSD related to mutated *NR5A1*. The patient presented with azoospermia since the first semen analysis when he was 16 years 4 months. A conventional TESE was performed at 17 years 10 months, but this procedure did not retrieve sufficient sperm cells for cryopreservation to perform an ICSI for future parenthood. These data extend the knowledge regarding fertility in *NR5A1* mutated patients. Further investigations in *NR5A1* mutated patients would help define a fertility care protocol in order to increase their chances of fertility.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics committee of Lyon University Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

JT wrote the manuscript. IP, DM, FD, and FR-B supervised the laboratory procedures. JT, DM, LR, FD, FR-B, and IP interpreted the data. Patient care was performed by CG, EL, PB, SGD'E, BC, and IP. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human immature testicular tissue organ culture: a step towards fertility preservation and restoration

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Background: Cryopreservation of immature testicular tissue (ITT) is currently the only option to preserve fertility of prepubertal patients. Autologous transplantation of ITT may not be safe or appropriate for all patients. Therefore, methods to mature ITT *ex vivo* are needed.

Objectives: Aim to investigate the feasibility of inducing *in vitro* spermatogenesis from ITT cryopreserved for pediatric patients prior to initiation of gonadotoxic therapy.

Materials and methods: Cryopreserved-thawed ITT from prepubertal and peripubertal patients were cultured for 7, 16, and 32 days in medium with no hormones or supplemented with 5 IU/L FSH, 1 IU/L hCG, or 5IU/L FSH+1 IU/L hCG. Samples were evaluated histologically to assess tissue integrity, and immunofluorescence staining was performed to identify VASA (DDX4)+ germ cells, UCHL1+ spermatogonia, SYCP3+ spermatocytes, CREM+ spermatids, SOX9+ Sertoli cells. Proliferation (KI67) and apoptosis (CASPASE3) of germ cells and Sertoli cells were also analyzed. Sertoli and Leydig cell maturation was evaluated by AR and INSL3 expression as well as expression of the blood testis barrier protein, CLAUDIN11, and testosterone secretion in the culture medium.

Results: Integrity of seminiferous tubules, VASA+ germ cells and SOX9+ Sertoli cells were maintained up to 32 days. The number of VASA+ germ cells was consistently higher in the peripubertal groups. UCHL1+ undifferentiated spermatogonia and SOX9+ Sertoli cell proliferation was confirmed in most samples. SYCP3+ primary spermatocytes began to appear by day 16 in both age groups. Sertoli cell maturation was demonstrated by AR expression but the expression of CLAUDIN11 was disorganized. Presence of mature and functional Leydig cells was verified by INSL3 expression and secretion of testosterone. Gonadotropin treatments did not consistently impact the number or proliferation of germ cells or somatic cells, but FSH was necessary to increase testosterone secretion over time in prepubertal samples.

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Conclusion: ITT were maintained in organotypic culture for up to 32 days and spermatogonia differentiated to produce primary spermatocytes in both pre- and peripubertal age groups. However, complete spermatogenesis was not observed in either group.

KEYWORDS

in vitro spermatogenesis, male fertility preservation, testicular tissue cryopreservation, immature testicular tissue, gonadotoxic therapy, cancer survivorship

Introduction

Treatments for childhood cancers are improving with post-therapy survival rates of 88% in 2021 (1), which means most young patients can look forward to a full and productive life after cure, including the possibility of having children. However, an unfortunate side effect of cancer therapy is the loss of fertility, since treatments target not only the rapidly dividing cancer cells, but also the proliferating germ cells in the gonads of those patients (2, 3). Infertility rates among childhood cancer survivors can range from 42-66% (4). Young adult survivors of childhood cancers experience distress about the potential for infertility (5–7), and desire to have offspring from their own cells, especially those with the religious restrictions of using donated sperm in some cultures (8). Therefore, the American Society for Clinical Oncology (9), the American Society for Reproductive Medicine (10) and the International Society for Fertility Preservation (11) recommend that all patients are counseled about the reproductive side effects associated with treatment of their primary disease as well as options to preserve fertility. Adult patients have the option to cryopreserve oocytes or sperm prior to treatment that can be thawed in the future and used to achieve pregnancy with established assisted reproductive technologies (12–14). The only fertility preservation options available to prepubertal children who are not yet producing mature oocytes or sperm are ovarian tissue or testicular tissue cryobanking (15, 16).

Although immature testicular tissue (ITT) from prepubertal boys do not produce sperm, they do contain spermatogonial stem cells (SSCs) that have the potential to produce sperm using one of several methods that are in the research pipeline (15). Autologous spermatogonial stem cell transplantation and testicular tissue grafting are mature technologies that have been replicated in numerous mammalian species (reviewed in (15)), and may be ready for translation to the human clinic. However, these autologous transplantation approaches may not be safe for patients with leukemia, testicular cancer or metastasizing disease due to concerns about reintroducing malignant cells back into patient survivors. Autologous transplantation may also be undesirable to transgender patients who do not want to be exposed to elevated testosterone levels that may be necessary to mature their tissues and produce sperm inside their bodies. Therefore, methods are needed to mature ITT outside the patient's body and produce sperm. Xenografting ITT into an animal host such as mice, pigs or primates, to mature and produce sperm is a promising option (17) but raises concerns about the potential risk of exposing patients to xenobiotic diseases (18). Furthermore, exposure of ITT to porcine or bovine hosts will not be acceptable in some religions (19, 20). Methods are needed to mature ITT outside the patient's body.

Sato and colleagues pioneered the method of ITT organotypic culture in 2011 in mice. Immature testicular tissue was cultured at the air/liquid interface on an island of agar that was half soaked in culture medium and matured over 30-40 days to produce sperm and live offspring (21). Over the past decade, the same group has continually optimized the approach using microfluidic devices to maintain tissues viable with continuous sperm production over several months (22–24). Critical features of the culture that supported long-term spermatogenesis in mouse organotypic culture were the replacement of FBS with knockout serum replacement (KSR) or Albumax, a lipid rich subfraction of KSR (23, 25, 26). Others have replicated the Ogawa testicular tissue organ culture methods with production of spermatids or sperm (27, 28) but production of offspring has not been replicated by any other research group in mice and translation to other species, including humans, remains a work in progress (see below).

Several groups have replicated the air/liquid interface culture with human ITT and reported variable results. Medrano and co-workers tested KSR versus FSH, 37°C versus 34°C, and with or without follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Menopur 75, Ferring) and reported that cultures maintained at 34°C with KSR exhibited the best survival of spermatogonia and Sertoli cells. The addition of LH and FSH improved Sertoli cell survival and promoted the maturation of germ cells up to the initiation of meiosis (SYCP3+ cells) but not beyond (29). Portela and colleagues used media supplemented with retinoic acid and melatonin, with or without FSH/LH. Spermatogonia survived and proliferated but the overall number of spermatogonia decreased over five weeks in culture and spermatogenesis was not initiated. Sertoli cells exhibited transient proliferation but did not mature to express the androgen receptor (AR). Culture outcomes were not impacted by cryopreservation or the addition of LH/FSH (30). Wang and colleagues used media supplemented with an extensive cocktail of growth factors, with or without retinoic acid (RA) (31). BOL+ spermatocytes were observed in the condition with RA on day 60 of culture, but those results were not quantified.

De Michele and colleagues used a variation of testis organotypic culture in which ITT was maintained in transwells at 34°C in an enriched DMEM-F12 medium supplemented with 10% KSR and 5 IU/L FSH. Viability and integrity of seminiferous tubules was maintained for 139 days, including the production of meiotic and post-meiotic cells. Spermatogonial proliferation and numbers decreased over time in culture. Sertoli cell numbers remained constant, but proliferation decreased over time in culture, which is typical of mature Sertoli cells, but AR expression did not change during culture. Leydig cell maturation was demonstrated by increased STAR expression and secretion of testosterone, which declined over time in culture. Supplementation of the culture medium with a cocktail of human chorionic gonadotropin (hCG), glial cell derived neurotrophic factor (GDNF), vitamin A and C, hydroxycholesterol, and triiodothyronine (T3) did not improve outcomes (32).

In summary, methods and results of organotypic culture with human immature testicular tissues are inconsistent and robust development of a complete seminiferous epithelium has not yet been achieved. Maturation of the testicular niche is likely critical to supporting complete spermatogenesis. LH and FSH are important for the maturation of Leydig cells and LH stimulates testosterone production, which is required for spermatogenesis. FSH supports the maturation of Sertoli cells that nurture every stage of spermatogenic lineage development and mediate the effects of testosterone (33–36). The studies described above included FSH and/or LH (or human chorionic gonadotropin, hCG) in some or all of their cultures but did not individually test the importance of these gonadotropins. The De Michele study reported the most advanced germ cell development with sporadic progression through meiosis and production of haploid cells. Therefore, we will attempt to replicate the De Michele approach (32, 37) and test the importance of FSH and LH, individually and in combination. Finally, we tested the hypothesis that Sertoli cells and Leydig cells in tissues from peripubertal patients are already mature and may be poised to support development of a spermatogenic epithelium.

Materials and methods

Human tissues

The UPMC Fertility Preservation Program (https://fertilitypreservationpittsburgh.org/) has cryopreserved >700 ITT since 2011 for patients who were at risk of infertility due to their diseases or medical treatments. Briefly, testicular tissues were obtained by a wedge biopsy comprising about 20% of one testis. Testicular parenchyma was dissected from any adhering tunica and cut into small pieces measuring 2-5 mm in diameter. Tissues are allocated 75% for the patient's future reproductive use and 25% to research; and cryopreserved in modified human tubal fluid containing 5% DMSO and 5% serum substitute supplement (SSS) (38). Research tissues were deidentified and transported to the research laboratory for cryostorage and experimentation (Figures 1A–C). All human subjects research was reviewed and approved by the University of Pittsburgh Institutional review board (IRB, STUDY19020220 and STUDY19110083) and registered with clinicaltrials.gov (NCT02972801).

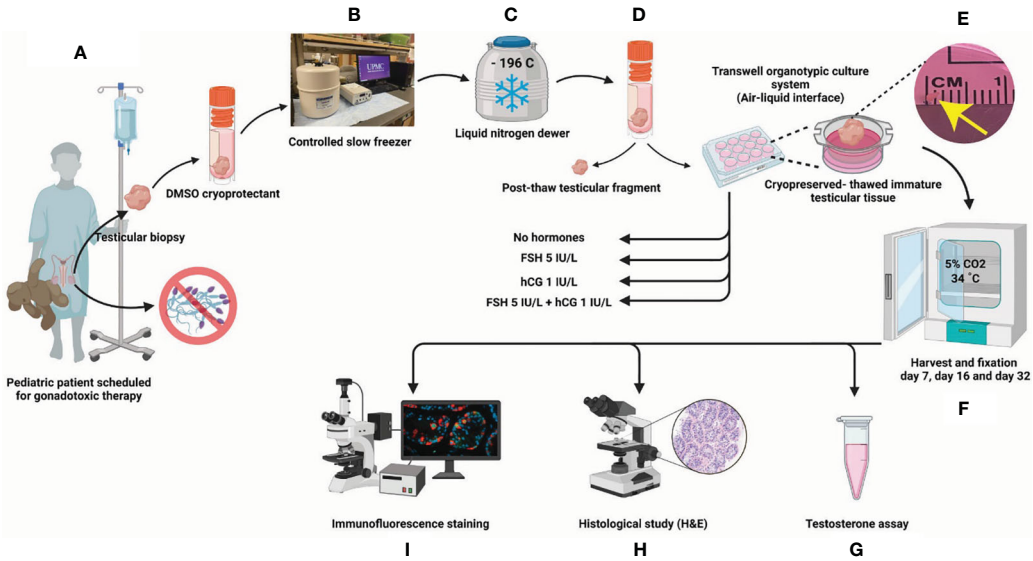

FIGURE 1
Experimental scheme. **(A)** ITT was collected by biopsy from patients before the initiation of gonadotoxic therapy and **(B)** cryopreserved by computer controlled slow rate freezing for **(C)** long term cryostorage in liquid nitrogen. Each patient donates 25% of their tissue to research. **(D)**. For this study, ITTs were thawed from three prepubertal patients (1-3 y/o) and three peripubertal patients (9-12 y/o). **(E)** ITT was cultured in DMEM/F12 media supplemented with 10% KSR and one of four *in vitro* treatments (none, FSH, hCG and FSH + hCG). **(F)** Cultured ITT was harvested on days 7, 16 and 32 of culture. **(G)** Culture media was assayed for testosterone. **(H)** Tissues were fixed for histology (hematoxylin and eosin) and **(I)** immunofluorescence.

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Study design

For the current study ITT from six patients was thawed as previously described (39) and allocated to prepubertal (ages 1, 2 and 3) and peripubertal (ages 9, 11 and 12) groups (Table 1). We excluded any patients who had previous gonadotoxic therapy, patients with potential malignant cells in their ITT (e.g., testicular cancer, leukemia) and patients with differences in sexual development (Table 1). Selected cryopreserved ITT samples were removed from liquid nitrogen dewars and thawed rapidly in a 37°C water bath, as described previously (39) (Figures 1C, D). Thawed testicular tissue for each patient was divided into five small fragments (~1 mm^3) when possible (see explanation for missing data in Supplementary Table 1). One ITT fragment was fixed in 4% paraformaldehyde (PFA) overnight and labeled as post-thaw sample and four fragments were placed in organotypic culture at the air-liquid interface as previously described by De Michele and colleagues (32). Briefly, ITTs were placed in 12 mm diameter/0.4 µm polycarbonate membrane Transwell® inserts (Corning® Incorporated) and cultured in a 5% CO2 humidified incubator at 34°C for 7, 16 or 32 days. The culture medium, CTS™ KnockOut™ DMEM/F-12 culture medium (Cat. No. A1370801, Thermofisher Scientific) supplemented with 10% CTS Knockout SR xenofree medium (KSR, Cat. No. 12618012, Thermofisher Scientific) and 1% penicillin-streptomycin (Cat. No. 15140122, Thermofisher, Gibco), was refreshed every 48 hours. ITT fragments of each patient were cultured under four conditions: 1) no gonadotropins, 2) 5 IU/L FSH (Gonal-F 75 IU, Merck Serono), 3) 1 IU/L hCG (Cat. No. CG5-1VL, lyophilized powder, vial of ~5,000 IU, Sigma-Aldrich) or 4) 5 IU/L FSH + 1 IU/L hCG, starting from day 2 (Figures 1D, E). Tissues from an individual patient were divided among the four gonadotropin treatment groups (no gonadotropins, FSH, hCG, FSH + hCG). There was not enough tissue from individual patients to also spread across the three culture timepoints. Therefore, one prepubertal sample (1 y/o) and one peripubertal sample (9 y/o) were analyzed on culture day 7; a second prepubertal (2 y/o) and second peripubertal (11 y/o) sample were analyzed on culture day 16 and a third prepubertal (3 y/o) and third peripubertal (12 y/o) sample were analyzed on culture day 32. Tissues from both pre- and peripubertal groups and each gonadotropin treatment group were collected on days 7, 16 and 32 of culture; fixed in 4% PFA overnight and paraffin embedded for histology and immunofluorescence staining (Figures 1F, H, I). Spent media was collected on days 4, 8 and 12 of culture and assayed for testosterone (Figure 1G). The testosterone experiment required additional samples because the d8 and d12 timepoints could not be collected from the cultures that were terminated on day 7 (Supplementary Table 2).

Histological analysis and immunofluorescence staining

Cultured testicular samples were harvested at each designated time point and fixed in 4% PFA overnight at 4C. The tissues were washed three times with D-PBS, embedded in paraffin and cut into 5 µm sections. Each tissue fragment was serially sectioned. A minimum of three non-consecutive sections (separated by 50 um or more) were analyzed for each experiment. Hematoxylin and eosin (H&E) staining was performed to assess tissue integrity under light microscopy. Integrity of seminiferous tubules was evaluated and divided into four scores as previously described (32, 39) (Figures 2A–D), where score 4 has the best tubular integrity and score 1 has the worst.

For immunofluorescence (IF) staining, the slides were warmed at 60°C and deparaffinized with 2X xylene, 5 minutes each. Sections were rehydrated in graded ethanol series: 2X 100% EtOH, 10 min; 95% EtOH, 5 min; 80% EtOH, 5 min; 70% EtOH, 5 min; 50% EtOH, 5 min; 25% EtOH, 5 min; PBS, 3 min. Antigen retreivel was performed in a 97°C water bath for 30 minutes using either sodium citrate (pH 6) or Tris (pH 10) buffers. Sections were blocked with 5% donkey serum for at least one hour and incubated with primary antibodies overnight. Different antibodies were used to characterize different stages and activities of germ and somatic cells. Goat anti-human DDX4 (VASA, 1:100, AF2030 Thermo Scientific) was used as a broad germ cell marker, goat or mouse anti-human UCHL1 (1:200, LS-B16043-50 or 7863-1004 Bio-Rad) for undifferentiated spermatogonia, rabbit anti-human SYCP3 (1:300, NB300-232SS Novus Biologicals) for primary spermatocytes, rabbit anti-human CREM (1:100, LS-B13702-50) for early spermatids, mouse anti-human KI67 (1:50 550609 BD Biosciences) for proliferation, rabbit anti-human cleaved CASPASE 3 (1:300, 96615 Cell Signaling Technology) for apoptosis, rabbit or goat anti-human SOX9 (1:400 Ab5535, Fisher Scientific or 1:100 AF3075 R&D) for Sertoli cells, rabbit anti-human AR (1:100 Ab271891 Abcam) for mature Sertoli cell, rabbit anti-human

TABLE 1 Patient groups for histology and IHC studies.

Pubertal category	Patient	Age (years)	Diagnosis	Previous treatment
Prepubertal	P1	1	Mucopolysaccharidosis (MPS)1	None
	P2	2	Chronic granulomatous disease	None
	P3	3	X linked chronic granulomatous disease	None
Peripubertal	P4	9	Rhabdomyosarcoma (prostate)	None
	P5	11	Chronic granulomatous disease	None
	P6	12	Ewing's sarcoma of fibula	None

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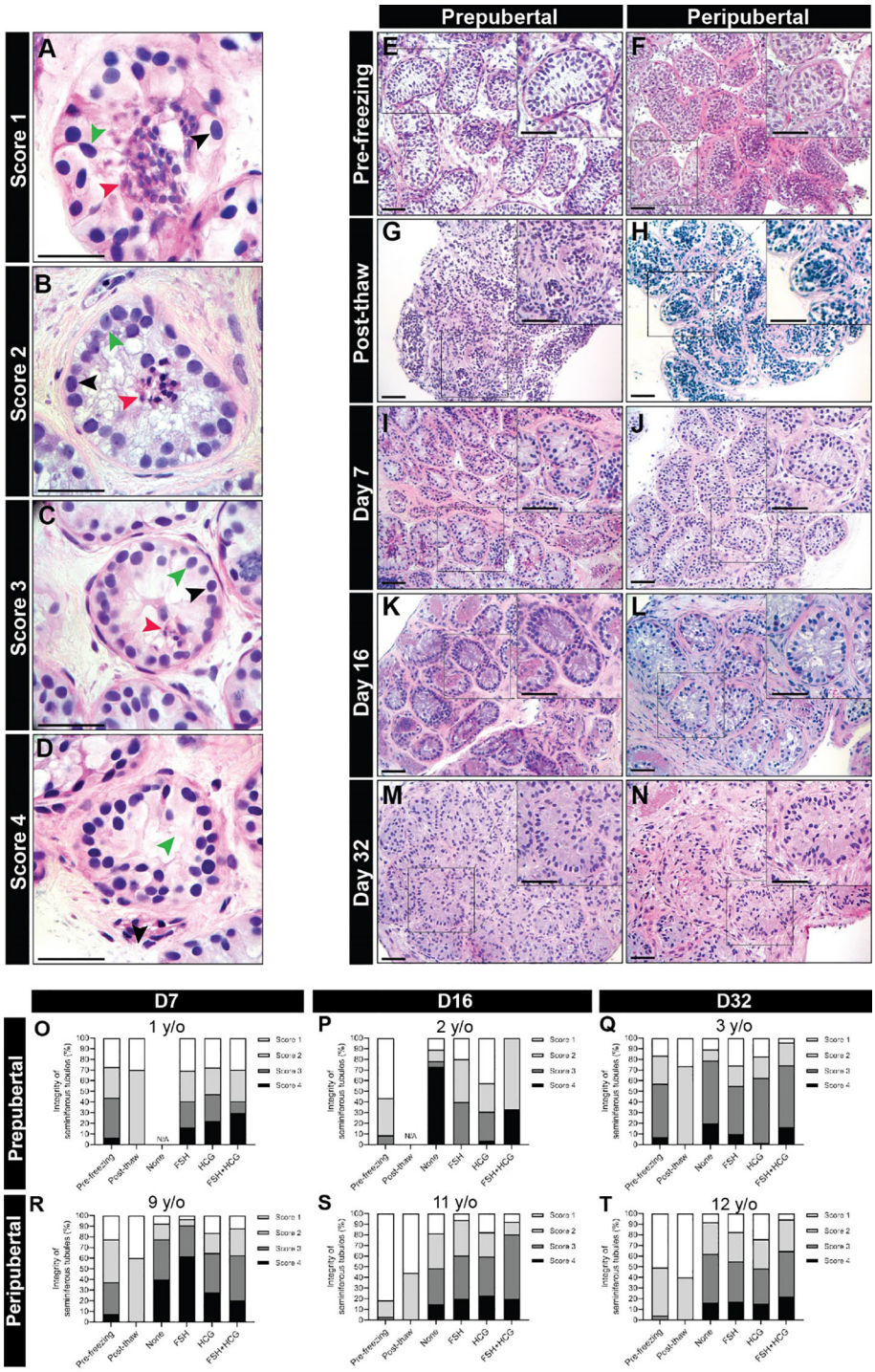

FIGURE 2
Scoring seminiferous tubule histological integrity. **(A–D)** Scoring system was used to classify the testicular samples as having poorly preserved **(A)**, score 1), fair **(B)**, score 2), good **(C)**, score 3) or best preserved **(D)**, score 4) histology. Black, green, and red arrowheads indicate spermatogonia, Sertoli cell, and pycnotic/apoptotic nuclei, respectively. Scale bars: 50 µm. **(E–N)** Hematoxylin and eosin-stained photomicrographs of immature testicular tissue from prepubertal and peripubertal patients at different time points: pre-freezing **(E, F)**, post-thaw **(G, H)**, and days 7 **(I, J)**, 16 **(K, L)** and 32 **(M, N)** *in vitro*. Scale bars: 100 µm. **(O–T)** Seminiferous tubules integrity classification based on scoring system from prepubertal **(O–Q)** and peripubertal patients **(R–T)** of pre- freezing, post-thaw, and four *in vitro* conditions (none, FSH, hCG and FSH + hCG), at different time points (day 7, 16 and 32).

CLAUDIN 11 (1:100 36-4500 Invitrogen) for tight junctions of blood-testis barrier and rabbit anti-human INSL3 (1:1500, HPA028615 Sigma) for mature Leydig cells. After 24 hours of incubation with primary antibodies, the slides were rinsed with Phosphate-buffered saline-Tween 20 (PBST) twice for 5 minutes each. Then the sections were incubated with secondary antibodies using Alexa Fluor® secondary antibodies conjugates 488, 568 or 647, (1:200, Invitrogen) for 45 minutes at room temperature. All

seminiferous tubules with a clearly demarcated basement membrane were included in the analyses without bias. An average 60 of cross-sections or longitudinal sections of seminiferous cords/tubules were measured, totaling an area of 947,000 +/- 496,000 µm2 per patient. Negative controls were stained with isotype control antibodies. Positive controls used adult human testicular tissues that contain complete spermatogenesis (Supplementary Figure 3). All slides were mounted with mounting medium containing DAPI (H-2000-10, Vector labs).

Testosterone assay

The supernatants of spent culture media were collected from 12 patients from both age groups at three time points: day 4, day 8 and day 12 and stored at -20C. Testosterone level was measured at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (https://med.virginia.edu/research-in-reproduction/ligand-assay-analysis-core/), using enzyme-linked immunosorbent assay (ELISA) (IBL-America).

Statistical analysis

For statistical analyses, all data are log2 transformed. For count data where 0 entries are present, 1 is added to all counts before the log2 transformation. All comparisons were based on linear regression models. When multiple comparisons were performed simultaneously, Tukey's *post hoc* tests were applied to control the familywise error rates.

Results

ITT histological evaluation

Seminiferous tubules from prepubertal and peripubertal groups showed a disorganized pattern post-thawing, with several pyknotic cells, indicated by lower integrity scores of 1-2 (compare pre-freezing in Figures 2E, F to post-thaw in Figures 2G, H). After 7 days of culture, germ and somatic cell organization and adherence to the basement membrane improved (Figures 2I-N), and high scores were assigned for all ITT fragments from 7-32 days *in vitro* (Figures 2O–T). We did not observe any obvious differences in the histology score of prepubertal versus peripubertal group during culture. Gonadotropin treatments did not appear to impact the histology score in the prepubertal or peripubertal groups during culture (Figures 2O–T).

Number of germ cells

Immunofluorescence staining confirmed the presence of VASA + germ cells in both prepubertal (Figure 3A) and peripubertal (Figure 3B) groups and that germ cells were maintained up to 32 days (Figures 3C-F). The number of germ cells in peripubertal tissue was significantly greater than in samples from prepubertal patients in almost every treatment group and at each culture timepoint (Figures 3C-F). Individual values for VASA+ cells in each treatment group at each culture timepoint, including post-thaw day 0 are in Supplementary Table 3. No consistent effect of gonadotropin treatment was observed in either group. There was a trend of decreasing germ cell numbers during the 32-day culture period, which was statistically significant in most culture conditions (Supplementary Figure 1).

Undifferentiated spermatogonia number, proliferation and apoptosis

UCHL1+ undifferentiated spermatogonia were observed in prepubertal and peripubertal tissues (Figures 4A-D) and their numbers were not consistently impacted by treatment or time in culture (Figures 4E-H). Proliferation (KI67+) and apoptosis (CASPASE 3+) of undifferentiated spermatogonia (UCHL1+) were assessed by immunofluorescent co-staining (Figure 5A). Proliferating spermatogonia (UCHL1+/KI67+) were observed in both age groups and all treatment conditions representing 0.5-30% of total UCHL1+ spermatogonia. Individual values for UCHL1+ cells in each treatment group at each culture timepoint, including post-thaw day 0 are in Supplementary Table 3. Gonadotropin treatments did not have a consistent effect on spermatogonial proliferation in either age group ($p>0.05$, Figures 5B-E). Caspase3 + apoptotic spermatogonia were rarely observed in in any culture condition or in either age group (data not shown).

Spermatogonial differentiation

Spermatogonial differentiation and initiation of meiosis was indicated by the appearance of SYCP3+ cells in most samples on day 16 and day 32 in both prepubertal and peripubertal ITT cultures (Figure 6). No SYCP3+ cells were observed in the pre-culture (post-thaw, Figure 6C) or day 7 culture samples (Figures 6E-H) in either age group. Individual values for SYCP3+/VASA+ cells in each treatment group at each culture timepoint, including post-thaw day 0 are in Supplementary Table 3. Gonadotropin treatment did not impact the appearance of SYCP3+ cells. We did not observe CREM+ early spermatids in either age group or any culture condition.

Sertoli cell number, proliferation and apoptosis

SOX9+ Sertoli cells were observed throughout the culture period and their numbers were not consistently impacted by age, gonadotropin treatment or time in culture (Figure 7). Individual values for SOX9+ cells in each treatment group at each culture timepoint, including post-thaw day 0 are in Supplementary Table 3.

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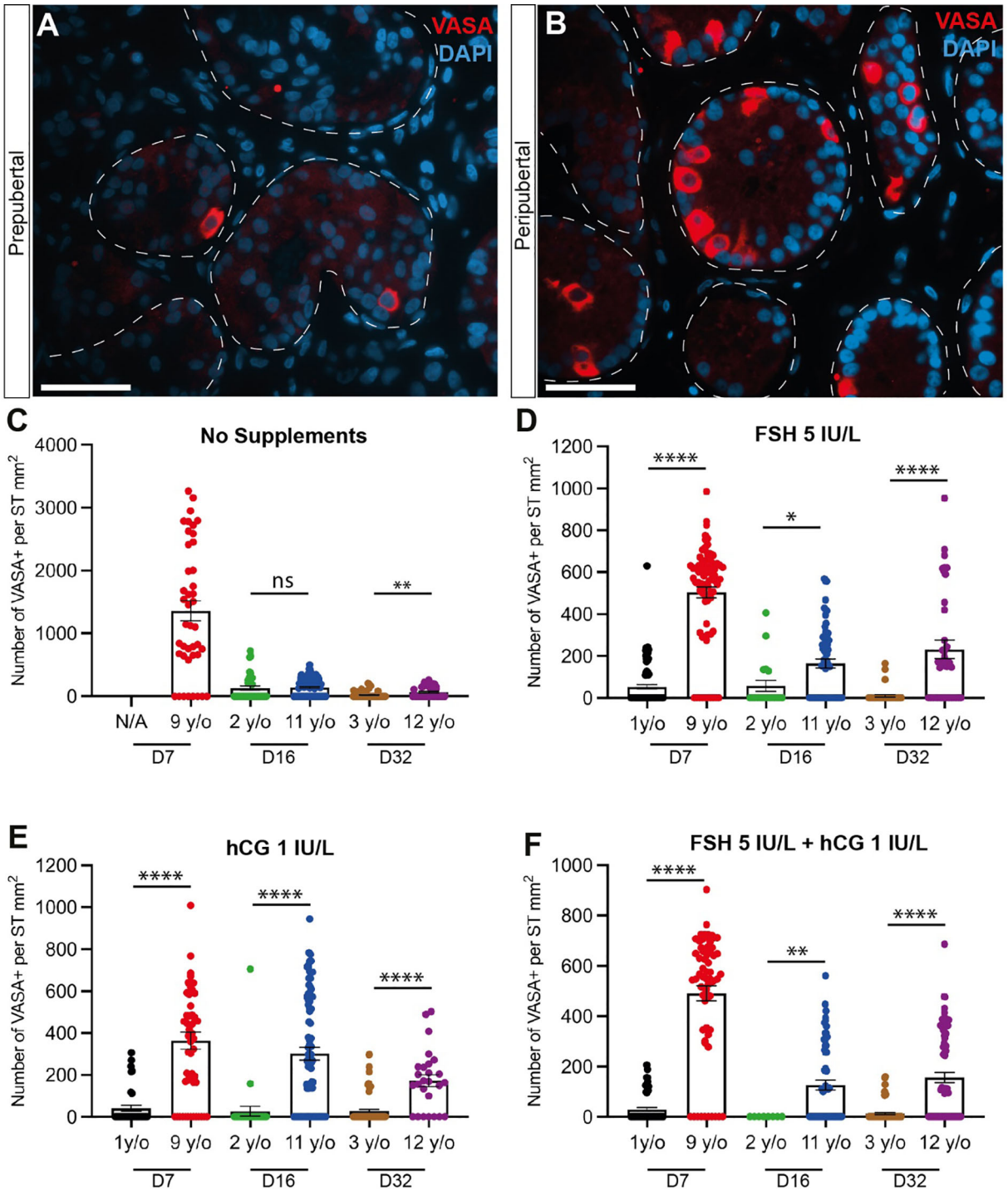

FIGURE 3
Quantification of germ cells. **(A, B)** Immunofluorescence staining for pan-germ cell marker, VASA in prepubertal **(A)** and peripubertal **(B)** age groups. Scale bars: 50 µm. Dashed white line: basal lamina. **(C–F)** Number of VASA+ germ cells per mm^2 of seminiferous tubule in prepubertal and peripubertal patients on day 7, 16 and 32 in four *in vitro* hormonal conditions, none **(C)**, FSH **(D)**, hCG **(E)** and FSH+ hCG **(F)**. Statistically significant differences are indicated (*p ≤ 0.05, **p ≤ 0.01, ****, p ≤ 0.0001). Raw data VASA+ germ cell numbers for post-thaw day 0 as well as days 7, 16 and 32 of culture are shown in Supplementary Table 3.

Gonadotropin treatments did appear to impact the number of SOX9+ cells, but the direction of impact was inconsistent within and between groups (Figures 6E-H), suggesting that changes may be due to individual variation among patient tissues. Proliferation (KI67+) and apoptosis (CASPASE3+) of Sertoli cells (SOX9+) were evaluated by immunofluorescence co-staining (Figures 8A, B). While the 1-year-old sample had a higher proportion of proliferating Sertoli cells in all treatment groups, there was no

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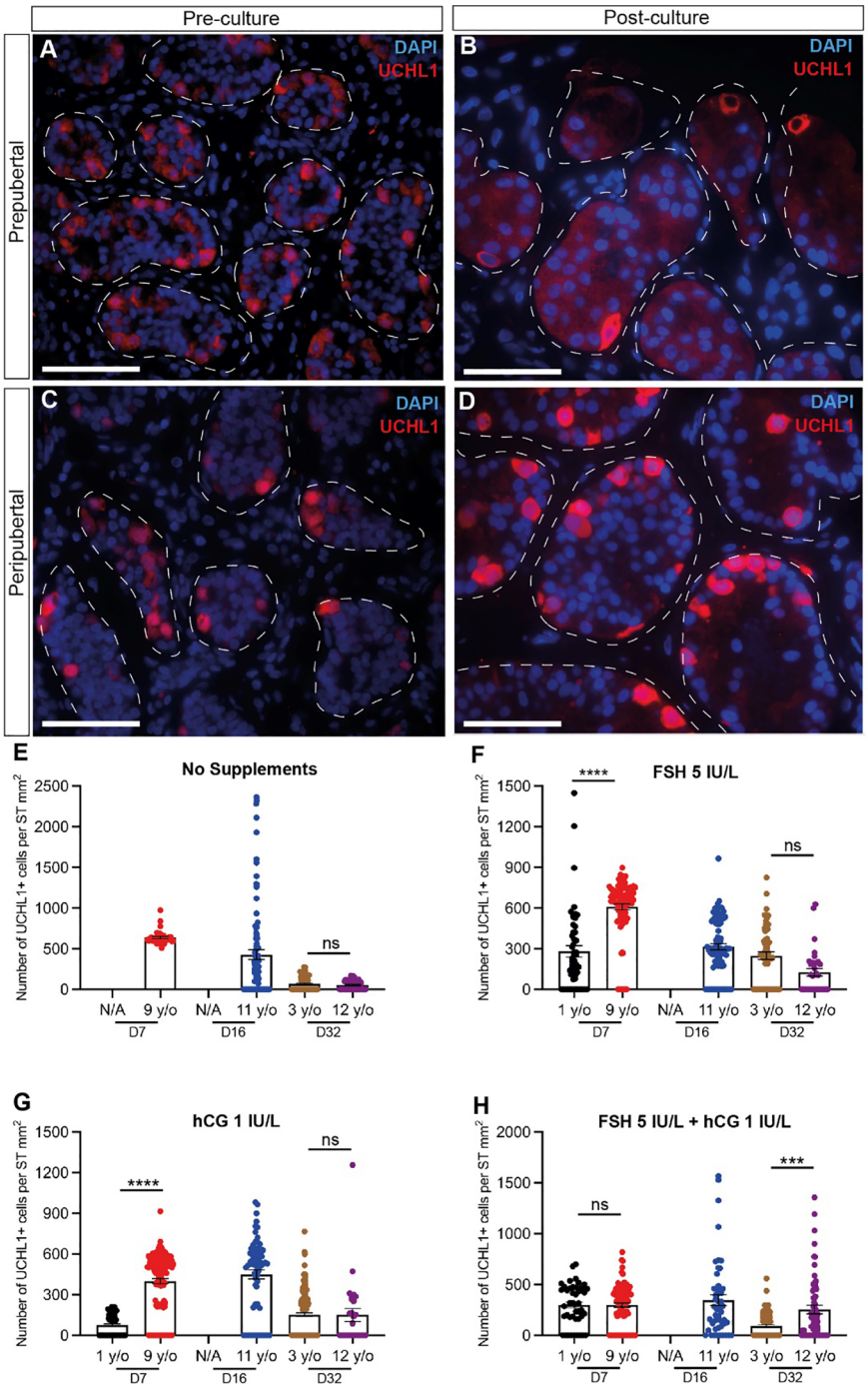

FIGURE 4
Quantification of undifferentiated spermatogonia. **(A–D)** Immunofluorescence staining for UCHL1+ undifferentiated spermatogonia in pre cultured **(A, C)** and post-cultured **(B, D)** ITTs in prepubertal **(A, B)** and peripubertal **(C, D)** groups. Dashed white line: basal lamina. Scale bars: 50 µm. **(E-H)** Number of UCHL1+ spermatogonial cells per mm² of seminiferous tubule in prepubertal and peripubertal patients on day 7, 16 and 32 of culture in four *in vitro* conditions, none **(E)**, FSH **(F)**, hCG **(G)** and FSH + hCG **(H)**. Statistically significant differences are indicated (***p ≤ 0.001, ****p ≤ 0.0001). Raw data UCHL1+ germ cell numbers for post-thaw day 0 as well as days 7, 16 and 32 of culture are shown in Supplementary Table 3.

consistent impact of gonadotropin treatment on the proportion of proliferating Sertoli cells (Figures 8C-F). The proportion of proliferating Sertoli cells decreased from day 7 to day 32 in both age groups and in all conditions (Figures 4B-H, Supplementary Figure 2). The number of CASPASE3+ apoptotic Sertoli cells was low in all culture conditions and both groups (data not shown).

Sertoli cell maturation

Sertoli cell maturation was assessed by immunofluorescence co-staining for SOX9 and androgen receptor (AR). There were fewer AR+ Sertoli cells in the prepubertal samples (Figure 9A) than peripubertal samples (Figure 9C) prior to culture. The number

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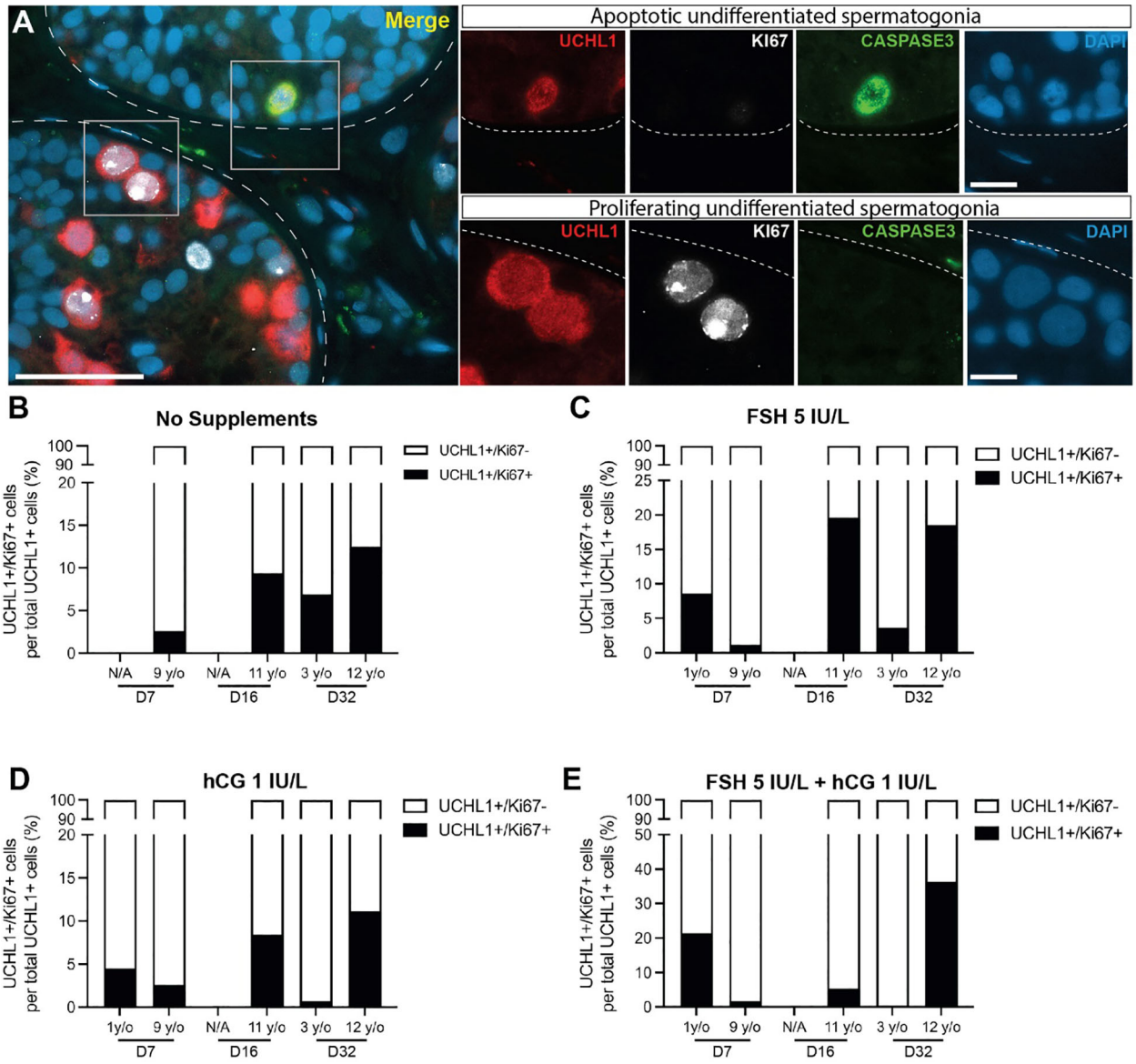

FIGURE 5
Proliferation and apoptosis of undifferentiated spermatogonia. **(A)** Immunofluorescence co-staining of UCHL1+ undifferentiated spermatogonia, proliferation marker, KI67, and apoptosis marker, CASPASE3. Dashed white line: basal lamina. Scale bars: 50 µm for A and 10 µm for high magnification single channel panel breakouts. **(B–E)** UCHL1+/KI67+ proliferating spermatogonia as a percentage of total UCHL1+ spermatogonia in prepubertal and peripubertal patients on day 7, 16 and 32 in four *in vitro* conditions, none **(B)**, FSH **(C)**, hCG **(D)** and FSH + hCG **(E)**. Raw quantitative data for the number of UCHL1+ spermatogonia in each treatment condition of the two age groups at different time points are shown in Figure 4.

(Figures 9B, D) and fluorescence intensity (Figures 9E-J) of AR+ cells increased significantly during culture in both age groups. Gonadotropin treatments did not consistently impact Sertoli cell AR expression intensity (Figures 9E-J).

Tight junction protein expression

CLAUDIN11 immunofluorescence was used to mark Sertoli cell tight junctions and the blood-testis barrier (BTB). CLAUDIN11 clearly delineated the BTB in adult tubules, separating the basal from the adluminal compartment of the seminiferous tubules (Figure 10A). In contrast, CLAUDIN11 was diffuse and did not delineate a BTB in the seminiferous tubules of ITT in either age groups, before (Figures 10B, D) or after (Figures 10C, E) culture.

Leydig cell maturation and functionality

INSL3+ Leydig cells were virtually absent in prepubertal, preculture tissues (Figure 11A), but appeared by day 32 in culture (Figure 11B). INSL3+ Leydig cells were present in peripubertal, preculture tissues (Figure 11C) as well as all culture days (Figure 11D). We also investigated Leydig cell functionality by measuring testosterone secretion into the media on days 4, 8 and 12 of culture. Testosterone levels in culture medium from both age

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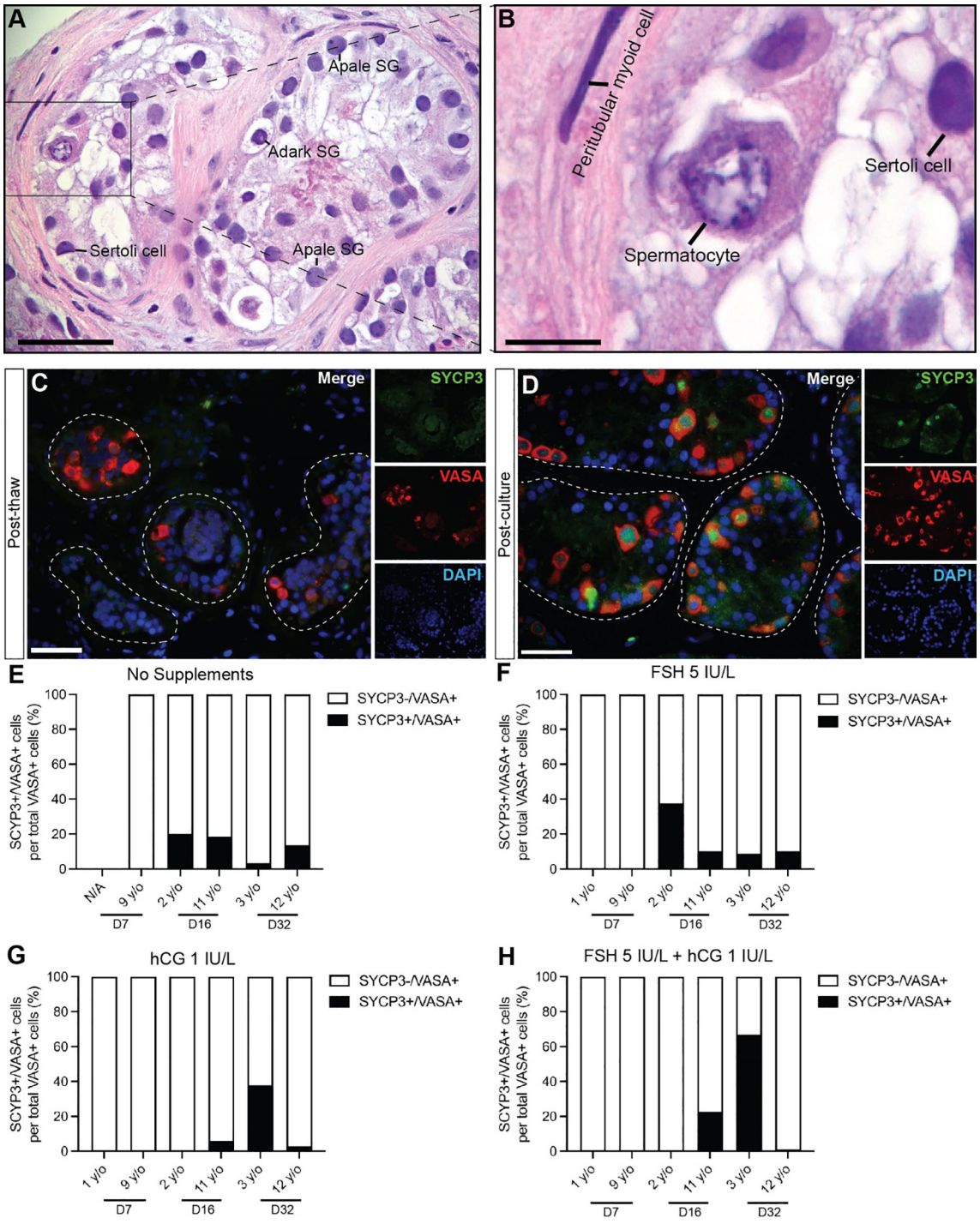

FIGURE 6
Spermatogonial differentiation. **(A, B)** Hematoxylin and eosin staining of cultured immature testicular tissue showing the presence of primary spermatocytes. Scale bars: 50 µm **(C, D)** Immunofluorescence of SYCP3+ primary spermatocytes and VASA+ germ cells of post thaw ITT **(C)** and post cultured ITT **(D)**. Dashed white line: basal lamina. Scale bars: 50 µm. **(E–H)** Percentage of SYCP3+ primary spermatocytes per total number of VASA+ germ cells in prepubertal and peripubertal patients on day 7, 16 and 32 in four *in vitro* conditions, none **(E)**, FSH **(F)**, hCG **(G)** and FSH + hCG **(H)**. Raw data VASA+/SYCP3+ germ cell numbers for post-thaw day 0 as well as days 7, 16 and 32 of culture are shown in Supplementary Table 3.

groups and in most treatment, conditions were higher on days 8 and 12 of culture than in pre-culture, day 0 or culture day 4 media (Figures 11E-L). The FSH treatment group had significantly higher levels of testosterone on days 8 and 12 than day 4 of culture. The hCG treatment group had higher testosterone levels on days 8 and 12 than day 4 in the peripubertal groups, but not the prepubertal group. The combination FSH + hCG group had higher testosterone levels on day 12 than day 4. There was no difference in testosterone levels between the two age groups in all conditions (data not shown).

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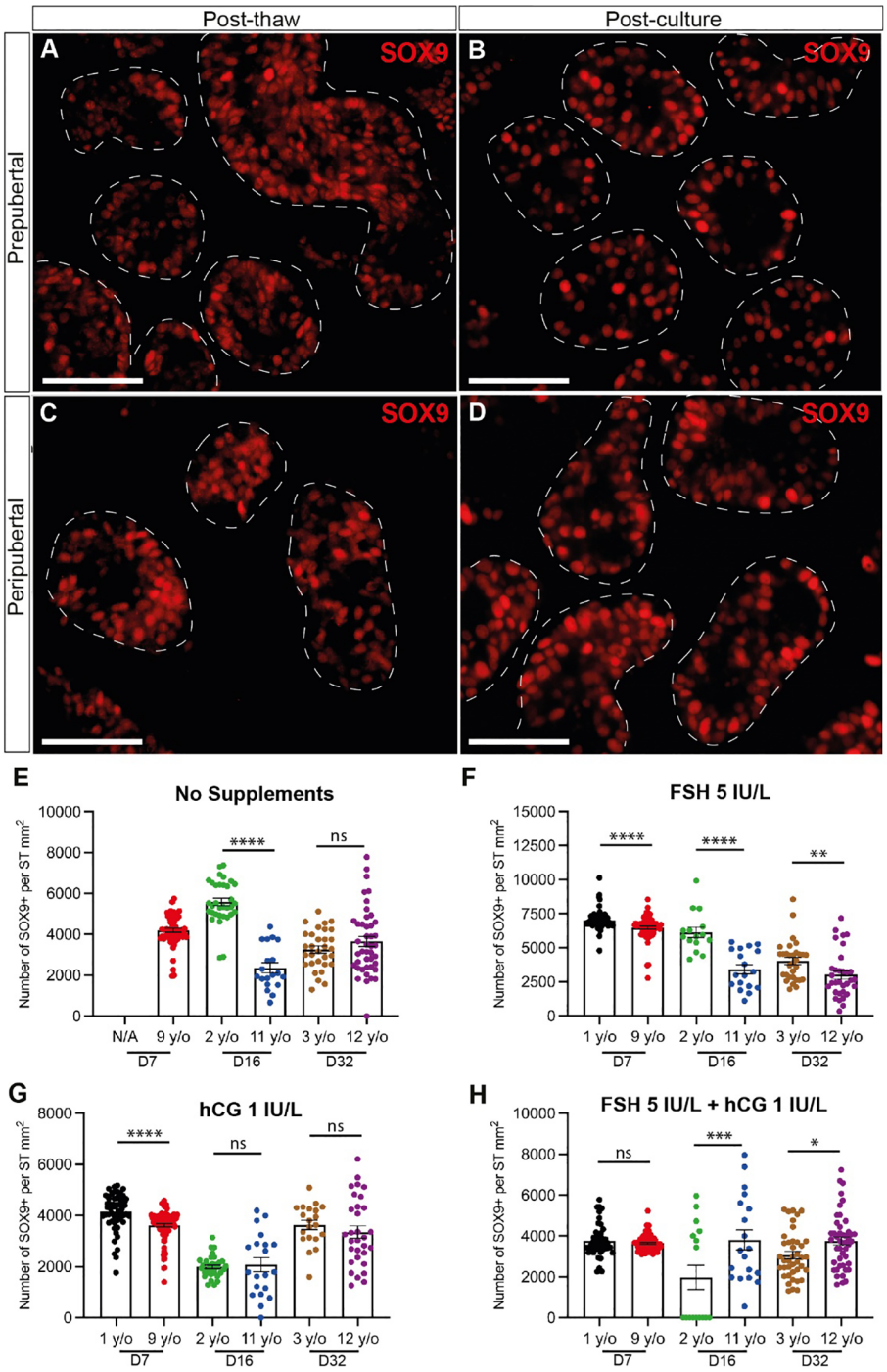

FIGURE 7
Quantification of Sertoli cells. **(A–D)** Immunofluorescence staining of SOX9+ Sertoli cells in post-thawed **(A, C)** and post-cultured **(B, D)** ITTs in prepubertal **(A, B)** and peripubertal **(C, D)** groups. Dashed white line: basal lamina. Scale bars: 50 µm. **(E–H)** Number of SOX9+ Sertoli cells per mm^2 of seminiferous tubule in prepubertal and peripubertal patients on day 7, 16 and 32 in four *in vitro* conditions, none **(E)**, FSH **(F)**, hCG **(G)** and FSH + hCG **(H)**. Statistically significant differences are indicated (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). Raw data SOX9+ germ cell numbers for post-thaw day 0 as well as days 7, 16 and 32 of culture are shown in Supplementary Table 3.

Discussion

Immature testicular tissue has been cryopreserved for thousands of patients worldwide, dating back to 2005 (40). Our center has cryopreserved testicular tissue for >700 patients since 2011 and some of those patients are returning to use their tissue for reproduction. Autologous spermatogonial stem cell transplantation and testicular tissue grafting are mature technologies that may be ready for the clinic (15), but those technologies will not be safe or appropriate for all patients. Organotypic culture is an *ex vivo* approach to mature ITT and produce sperm. The proof in principle for this approach in mice was initially published by Sato

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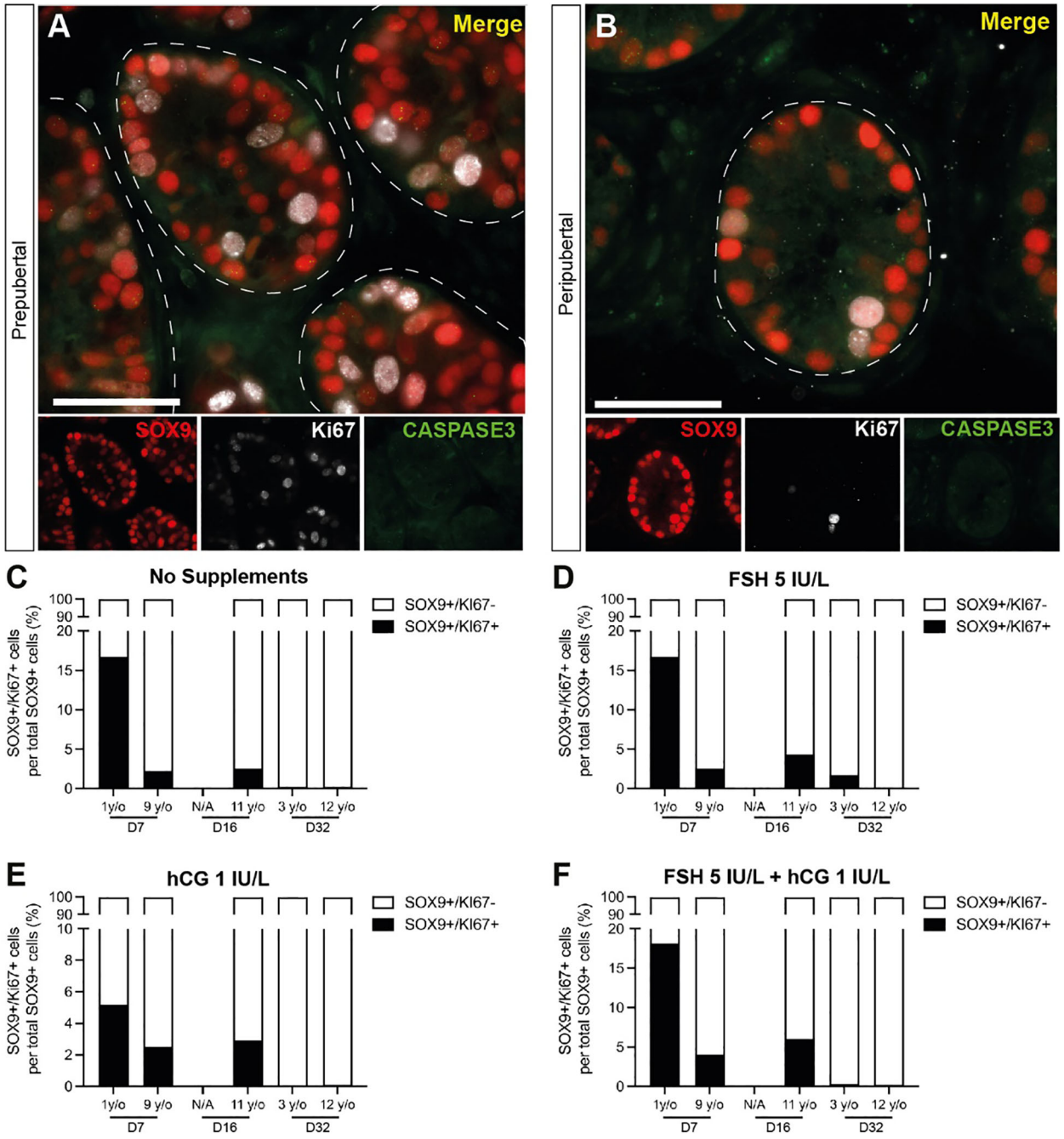

FIGURE 8
Proliferation and apoptosis of Sertoli cells. **(A, B)** Immunofluorescence co-staining of SOX9+ Sertoli cells with proliferation marker, KI67, and apoptosis marker, CASPASE3 in cultured ITTs of prepubertal **(A)** and peripubertal **(B)** groups. Dashed white line: basal lamina. Scale bars: 50 µm. **(C–F)** SOX9+/KI67+ proliferating Sertoli cells as a percentage of total SOX9+ Sertoli cells in prepubertal and peripubertal patients on day 7, 16 and 32 in four *in vitro* conditions, none **(C)**, FSH **(D)**, hCG **(E)** and FSH + hCG **(F)**. Raw quantitative data for SOX9+ Sertoli cells in each treatment condition are shown in Figure 7.

and colleagues in 2011 with the production of sperm and offspring (21). Several labs have attempted to replicate those results with human ITT, but progress has been limited. Human ITT can remain viable for times ranging from 7-139 days in culture. Spermatogonia survive and sporadically differentiate to produce spermatocytes or spermatids, but robust regeneration of a complete seminiferous epithelium has not been described (29–32). Previous studies of human ITT organotypic culture included FSH and/or LH in some or all of their culture conditions but did not independently and exclusively test the impact of those two gonadotropins.

The pubertal surge of gonadotropins is necessary to mature testicular somatic cells and create an environment that supports the initiation of spermatogenesis (33, 34, 41, 42). Similarly, we hypothesize that LH (hCG) and/or FSH are necessary to mature somatic cells in ITT and support spermatogenesis. Surprisingly, it does not appear that these gonadotropins have been independently

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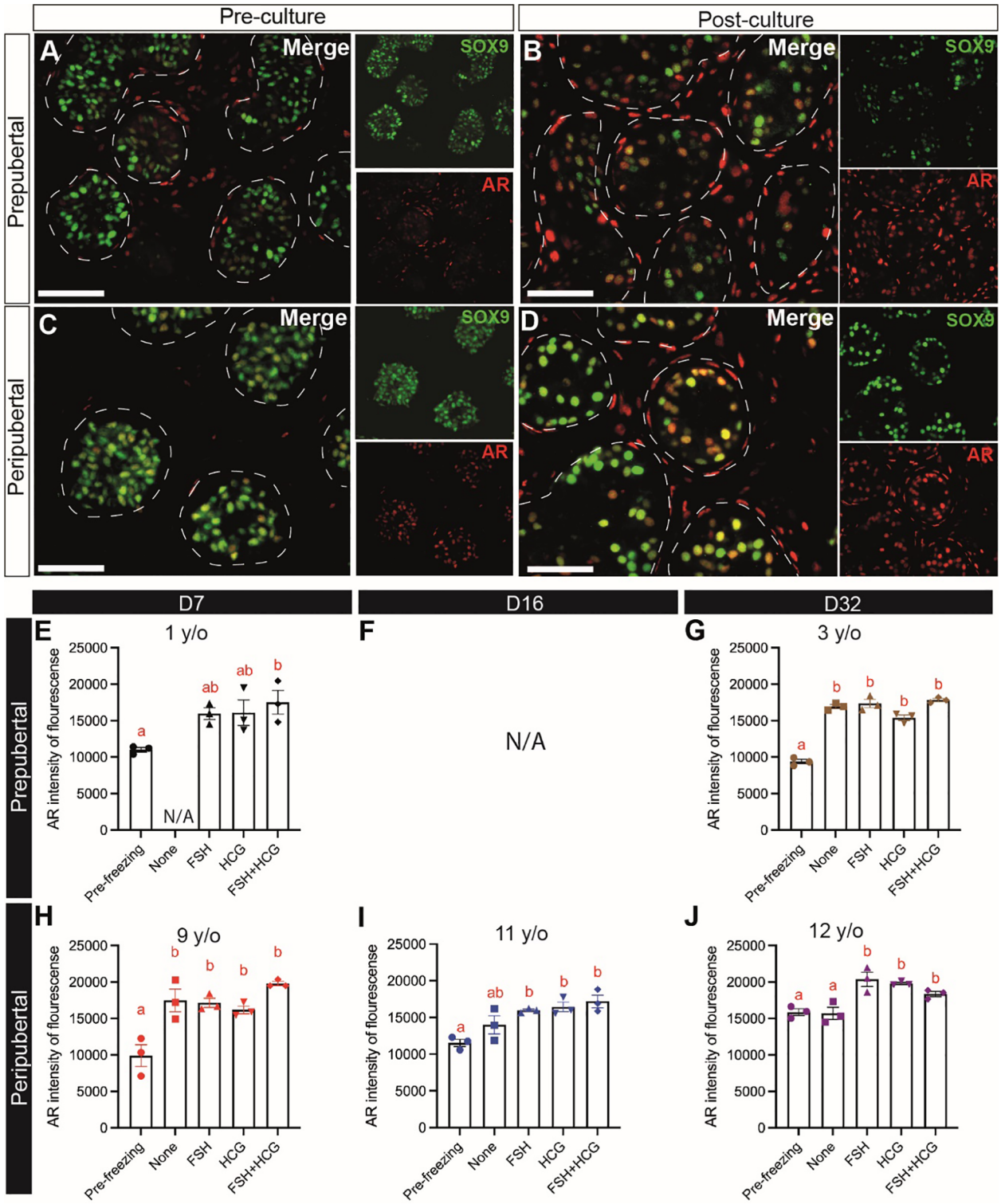

FIGURE 9
Sertoli cell maturation (AR expression). **(A–D)** Immunofluorescence co-staining of AR and SOX9+ Sertoli cells in pre-cultured **(A, C)** and post-cultured **(B, D)** ITTs in prepubertal **(A, B)** and peripubertal **(C, D)** groups. Dashed white line: basal lamina. Scale bars: 50 µm. **(E–J)** AR fluorescence intensity in prepubertal **(E–G)**, F is missing data, See Supplementary Table 1 for explanation) and peripubertal **(H–J)** patients in four *in vitro* conditions, none, FSH, hCG and FSH + hCG at days 7 **(E, H)**, 16 **(F, I)** and 32 **(G, J)** of culture. Different red letters above the bars indicate statistically significant differences between groups ($p \leq 0.05$).

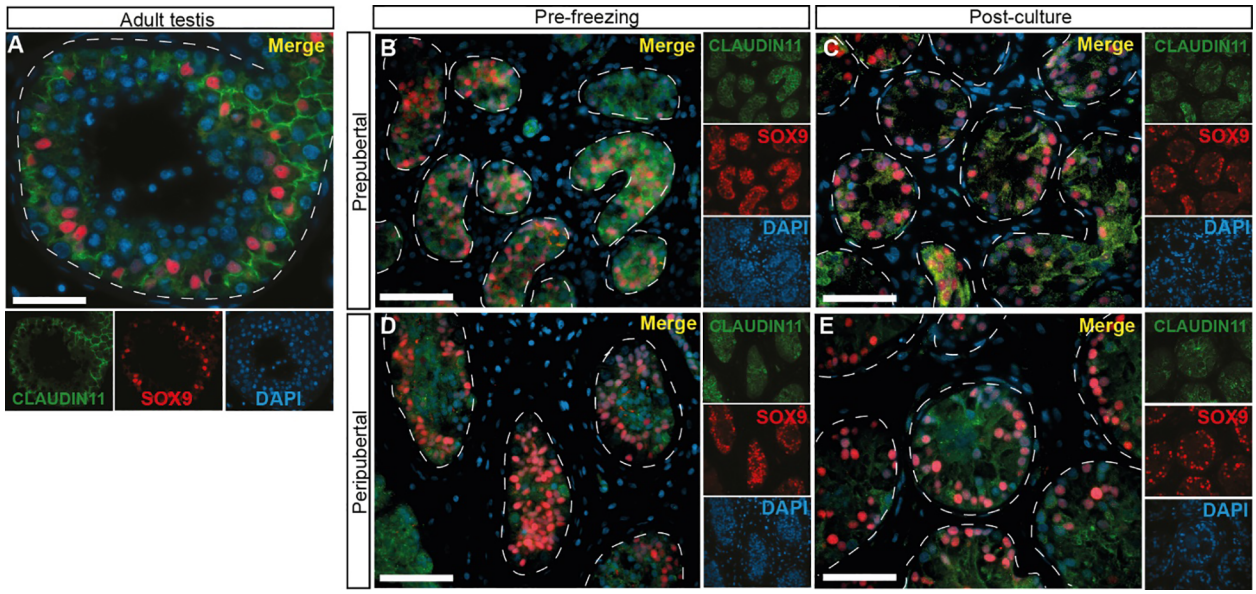

FIGURE 10
CLAUDIN 11 expression, a marker of Sertoli cell tight junctions and the blood-testis barrier. **(A–E)** Immunofluorescence staining for CLAUDIN11 and SOX9 in adult testicular tissue **(A)**, prepubertal **(B, C)** and peripubertal **(D, E)** ITTs before **(B, D)** and after **(C, E)** culture. Dashed white line: basal lamina. Scale bars: 50 µm.

tested in organotypic culture of human ITT in the absence of other hormones or growth factors. We replicated the transwell ITT culture system described by de Michele and colleagues because that system was the only one to report the appearance of haploid cells (32); and tested four hormonal conditions (no hormones, 5 IU/L FSH, 1 IU/L hCG, and 5IU FSH + 1 IU/L hCG). These are the gonadotropin doses used by de Michele and colleagues. We also compared culture outcomes from prepubertal versus peripubertal testicular tissues to test the hypothesis that testicular somatic cells may already be partially matured in the peripubertal testis.

Upon thawing of cryopreserved ITT, we found that integrity of seminiferous tubules was disrupted compared to the pre-cryopreservation histology. However, tubular integrity quickly improved in culture, with tubular integrity scores resembling the pre-cryopreservation tissues at all culture times and treatment groups. Tubular integrity was maintained for up to 32 days in culture (the longest time tested in this study). As expected, VASA+ germ cells were present in higher numbers in peripubertal tissues than prepubertal tissues. VASA+ cells in both age groups were maintained throughout the culture period but their numbers declined over time in culture, as reported by others (29–32). However, gonadotropin treatments in any combination increased the number of germ cells remaining in peripubertal patient samples compared to the no treatment group, except for the 9-year-old patient, who had a higher number of germ cells in all treatment conditions. Gonadotropin treatment did not impact the number of VASA+ germ cells in prepubertal samples, perhaps suggesting that prepubertal somatic cells were not yet competent to respond to gonadotropins. Prepubertal samples tested in this study were from 1, 2 and 3 year old patients. Future studies should test older prepubertal stages (e.g., 4-8) to learn when prepubertal testicular somatic cells acquire the competence to respond to gonadotropins and dissect the molecular mechanisms that regulate that transition. Similar to VASA+ cells, UCHL1+ undifferentiated spermatogonia were present and proliferating throughout the 32 day culture period, which again, is consistent with previous reports (30, 32). Gonadotropin treatments did not appear to impact the number or proportion of proliferating spermatogonia in either age group. This is in contrast to the observations of Medrano and colleagues, who found that supplementation with gonadotropins (FSH and LH) increased the number of UTF1+ undifferentiated spermatogonia (29). Those differences may be attributed to the fact that UTF1 is a more restricted marker of undifferentiated spermatogonia, while UCHL1 has a broader expression profile that also includes some differentiating spermatogonia (43).

SYCP3 and CREM were used to assess spermatogonial differentiation to spermatocytes and spermatids, respectively. Neither marker was present in pre-culture tissues or day 7 cultures from either age group. SYCP3+ spermatocytes began to appear in both age groups on days 16 and 32 of culture, which is consistent with previous reports, *in vivo* and *in vitro* (32, 44, 45). There was no consistent effect of gonadotropin treatments on the appearance of SYCP3+ cells. We did not observe CREM+ spermatids in either age group, at any time of culture in any of

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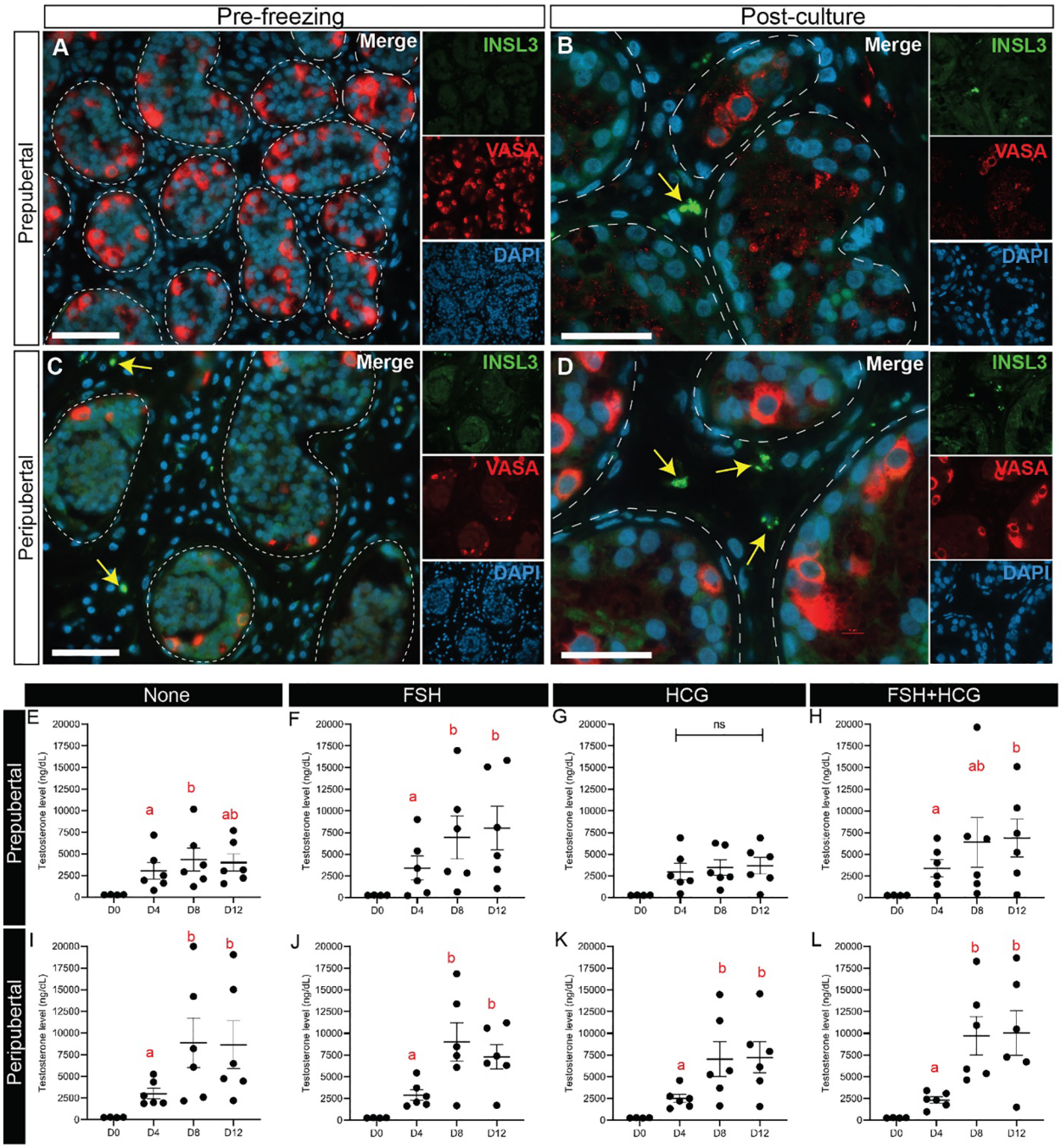

FIGURE 11
Leydig cell maturation and testosterone secretion. **(A-D)** Immunofluorescence staining for INSL3 and VASA in prepubertal **(A, B)** and peripubertal **(C, D)** patients before **(A, C)** and after **(B, D)** culture. Dashed white line: basal lamina. Scale bars: 50 µm. **(E-L)** Testosterone levels (ng/dL) in prepubertal **(E-H)** and peripubertal **(I-L)** patients in four *in vitro* conditions, none **(E, I)**, FSH **(F, J)**, hCG **(G, K)** and FSH + hCG **(H, L)** and on days 4, 8 and 12 of culture. Different red letters above the bars indicate statistically significant differences between groups ($p \leq 0.05$).

the culture conditions. Perhaps that is not surprising, since the process of spermatogenesis from spermatogonia to sperm takes 74 days in humans (46) and we did not extend our cultures to 74 days. Extended culture periods may allow sufficient time for progression through meiosis and production of haploid cells. We note, however, that de Michele and colleagues (32), using the same culture system, observed haploid cells by chromatin *in situ* hybridization on days 16, 32, 64 and 139 of ITT organotypic culture. The authors noted, however, that ACE+ spermatids were observed by immunohistochemistry only in one out of 40 sections in a 64-day culture from one patient and expression of late spermatid markers TP1 and PRM2 were not observed (32). While some studies indicated that spermatogenesis occurs on schedule in rodent organotypic culture (21, 47, 48), others have suggested that *in vitro* spermatogenesis may occur at an accelerated pace in mouse and humans (48, 49).

Sertoli cells are the testicular somatic cells of the seminiferous tubules that switch from immature and proliferative cells to mature and non-proliferative status around the time of puberty under the control of FSH and testosterone (34). They are considered the nurse cells of the adjacent germ cells since they orchestrate every stage of germ cell development from the most undifferentiated spermatogonia to the most differentiated spermatozoa. Sertoli cells also mediate many of the effects of testosterone on germ cell development (36). We found that SOX9+ Sertoli cell numbers, normalized to area of seminiferous tubule, were present in similar numbers in prepubertal and peripubertal samples and maintained throughout the 32 day culture period. Sertoli cell numbers were not impacted by gonadotropin treatment. This is consistent with the results of de Michele and colleagues who found that Sertoli cell numbers were maintained in long-term culture in the presence of FSH or FSH + hCG and other factors (32); but differs from the results of Medrano and colleagues, who found that Sertoli cell numbers decreased by 70 days in culture in the presence of FSH and hCG. While Sertoli cell number remained relatively constant throughout the culture period in our study, Sertoli cell proliferation decreased over time in both age groups, which may be an indicator of Sertoli cell differentiation. Sertoli cell differentiation was also indicated by increased AR expression during culture, consistent with previous reports (31, 32), and this occurred in a gonadotropin independent manner. Portela and colleagues did not observe induction of AR expression in ITT organotypic culture. Moreover, maturation of Sertoli cells in our study was likely incomplete because CLAUDIN11 proteins did not organize to delineate a BTB as shown in the adult control (Figure 10A). A functional BTB is necessary for the maintenance of spermatogenesis, *in vivo* (50). Others have also reported expression of BTB proteins (ZO-1, CLAUDIN11, CONNIXIN43) in ITT organotypic culture. Medrano and colleagues reported that ZO-1 expression pattern was chaotic (29). de Michele and colleagues reported that CLAUDIN 11 expression was constant for all patients at all culture times while CONNEXIN 43 expression was observed from day 16 onwards (51). *in vitro*

INSL3 is a constitutive marker of Leydig cell differentiation (52). As expected, we found that INSL3 expression was absent in prepubertal testis tissues, but present in peripubertal testis tissues prior to culture. INSL3 expression was observed in the testicular interstitium of both prepubertal and peripubertal tissues by 32 days in culture and this was not gonadotropin dependent (not shown). The number of INSL3+ cells was not quantified in this study but appeared fewer in number in the cultured samples than in the positive control (Supplementary Figure 3), perhaps indicating that Leydig cell differentiation was incomplete. Nonetheless, all tissues in all treatment conditions secreted testosterone into the culture medium, which increased over time in culture in most samples. The exceptions were untreated and hCG treated prepubertal tissues that did not exhibit increased testosterone secretion over time. This may indicate that FSH was needed to mature the Leydig cells and enhance responsiveness to hCG. This interpretation is consistent with a previous report of Kerr and Sharpe (35), who reported that FSH and not LH was required to mature Leydig cells and enhance hCG stimulated testosterone production in whole testes or dispersed Leydig cells.

Conclusion

We found that cryopreserved ITT from patients could be maintained in organotypic culture for up to 32 days. Testicular germ cells and somatic cells remained viable throughout the culture period. We observed sporadic differentiation of spermatogonia to produce SYCP3+ spermatocytes but did not observe differentiation to CREM+ meiotic or post-meiotic cells and did not observe the establishment of a complete seminiferous epithelium. Our study revealed differences in prepubertal and peripubertal tissues in terms of somatic differentiation and responsiveness to gonadotropins (testosterone production), but these differences did not impact the extent of spermatogenesis achieved in culture. Higher doses of gonadotropins and/or longer culture periods may support more complete spermatogenic lineage development in future studies. Somatic differentiation, especially of prepubertal tissues, occurred during culture (increased AR and INSL3 expression), but differentiation of Sertoli cells and Leydig cells may have been incomplete, which may have impacted the extent of spermatogenesis achieved in this study. The main limitations in this study were the low number of patients and the tiny size of each ITT fragment available to research. This made it impossible to spread the tissue from a single patient across all treatment groups and all culture timepoints. Each timepoint within each treatment group is represented by only one patient. Therefore, some of the observed differences were due to variations among individual patients, which may have obscured treatment effects. Nonetheless, our results were consistent with previous reports indicating that ITT can be maintained in culture while highlighting limitations that will be the focus of ongoing studies to achieve complete and robust spermatogenesis in cultured human testicular tissues.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by University of Pittsburgh Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal study was approved by University of Pittsburgh Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NY, SA and KO designed the experiments. NY conducted the experiments and wrote the manuscript. NY, AC-B, SA and KO analyzed and interpreted the results. TC conducted statistical analysis. NY, AC-B, SA and KO revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1242263/full#supplementary-material

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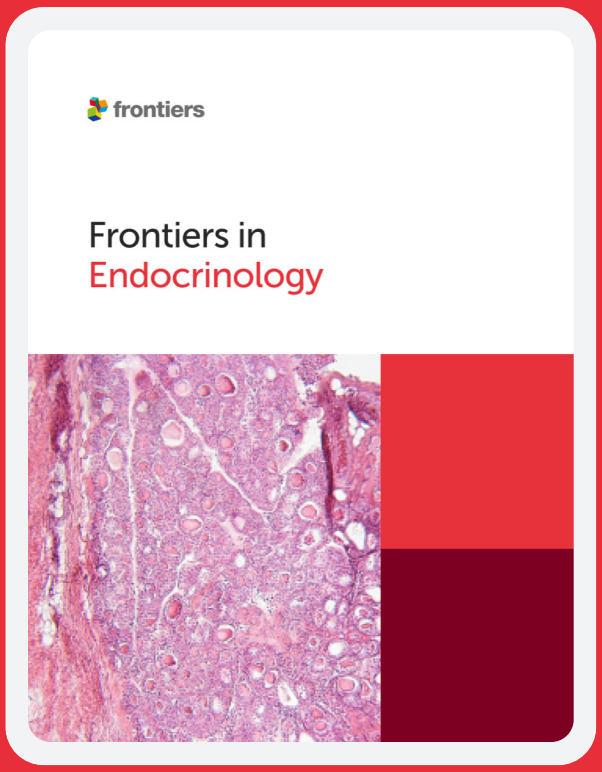

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