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Fertility preservation in the pediatric population

Edited by
Asma Chattha, Mahmoud Salama and Yasmin Jayasinghe

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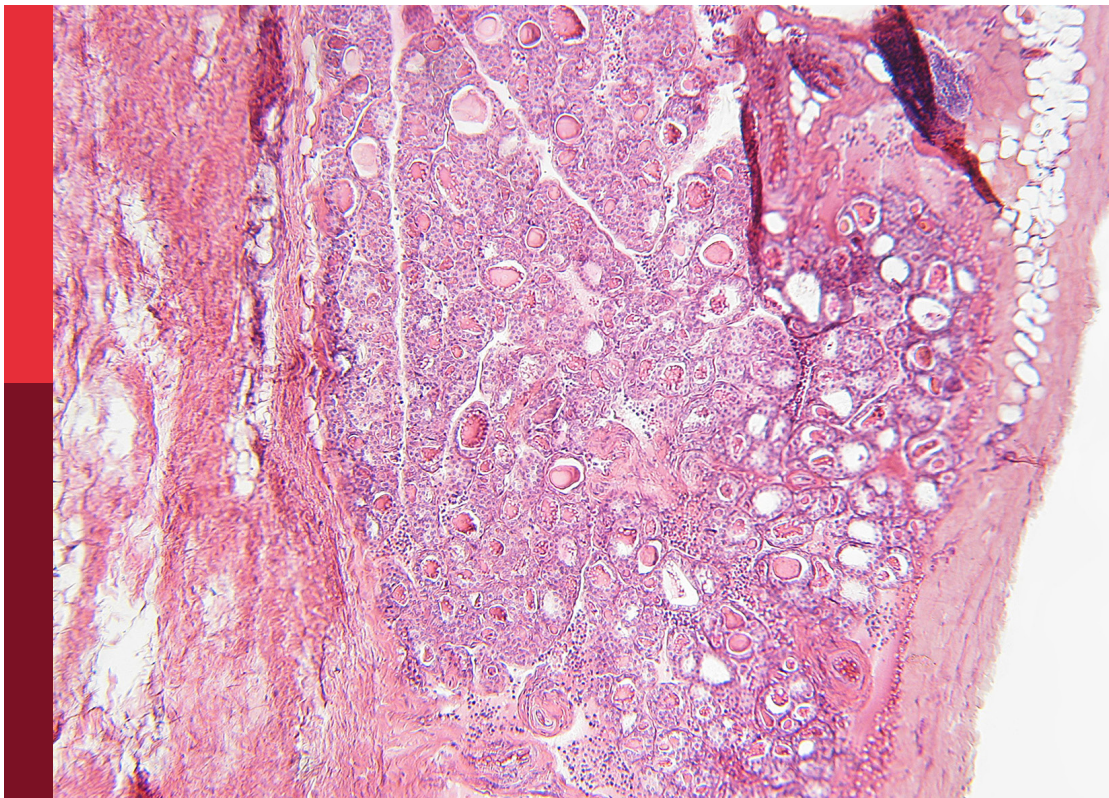

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Fertility preservation in the pediatric population

Topic editors
Asma Chattha — Mayo Clinic, United States
Mahmoud Salama — Michigan State University, United States
Yasmin Jayasinghe — The University of Melbourne, Australia

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Rutgers, The State University of New Jersey, United States

*CORRESPONDENCE
Asma J. Chattha
Chattha.Asma@mayo.edu

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Editorial: Fertility preservation in the pediatric population

Asma J. Chattha[1]*, Mahmoud Salama[2] and Yasmin Jayasinghe[3,4,5]

[1]Department of Pediatric and Adolescent Medicine, Mayo Clinic, Rochester, MN, United States, [2]Department of Obstetrics, Gynaecology and Reproductive Biology, Michigan State University, East Lansing, MI, United States, [3]Department of Obstetrics and Gynaecology, University of Melbourne, Royal Women's Hospital, Parkville, VIC, Australia, [4]Oncofertility Program, Royal Children's Hospital, Melbourne, VIC, Australia, [5]Murdoch Children's Research Institute, Melbourne, VIC, Australia

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pediatric, adolescent, oncofertility, fertility preservation, ovarian tissue cryopreservation, testicular tissue cryopreservation, transgender, disorders of sexual differentiation (DSD)

Editorial on the Research Topic
Fertility preservation in the pediatric population

The Frontiers in Endocrinology Research Topic on fertility preservation in children invited authors from across the globe to participate in the dissemination of knowledge and awareness regarding the best fertility preservation principles in the pediatric population. Although long considered a problem only in adults and post pubertal individuals undergoing cancer therapy, assisted reproductive technologies have rapidly advanced to include ovarian and testicular tissue preservation. This now allows prepubertal patients and families who were previously excluded from fertility conversations, to be included in these profoundly important discussions, which may provide hope for future attempts at parenthood (1). Fertility preservation is now considered for any medical condition requiring gonadotoxic treatment with curative intent, as well as those causing premature gonadal decline. This means that oncofertility care is now being rapidly expanded to include children with non-oncologic conditions affecting fertility such as genetic, rheumatologic, nephrologic disease, and hematologic conditions requiring bone marrow transplant, as well as the transgender population (1). However, many knowledge gaps exist in the pediatric population, which this Research Topic sought to address.

Disparities in oncofertility care across the globe are well described, both in high and low resource settings (2, 3). Many centers lack best practice oncofertility guidelines for children facing fertility-threatening diagnoses and treatment plans, resulting in significant distress for survivors (4). Furthermore, different aspects of oncofertility care are in different stages of translation. Ovarian tissue preservation has transitioned into standard practice, but requires ongoing monitoring in the young, while testicular tissue preservation is still experimental in humans (5, 6).

In this Research Topic, authors were invited to present their research on optimal methods, timing, and outcomes on fertility preservation in children and adolescents. Data on new populations eligible for fertility preservation is highlighted in this Research Topic.

Barrett et al. describe successful oocyte cryopreservation in 19 out of 20 transmen aged 12–20 years (median age 17 years).This is an important study since much of the previously published data is derived from the adult population. Two participants had been on testosterone, which was discontinued during oocyte collection. Around two thirds of patients cryopreserved at least 10 mature oocytes with many patients additionally freezing

immature oocytes. There was no difference in outcome in those who attempted oocyte cryopreservation who had been on oral contraception, puberty blockers, or testosterone, compared with those who were naïve to hormonal therapy. Importantly, the use of dysphoria protection protocols in accordance with the World Professional Association for Transgender Health were implemented (7). This included using appropriate language and pronouns, avoiding triggering terminology, avoiding pelvic examination, and utilizing transabdominal ultrasound monitoring in the majority of cases. Similar to other studies on children, this study highlighted the high desire for family building in the gender diverse population and the high uptake of fertility preservation when barriers are reduced and culturally sensitive care is provided (8).

Highlighting the need to expand fertility preservation to conditions outside cancer therapy, two very important articles focus primarily on fertility preservation practices in pediatric patients with hemoglobinopathies and disorders of sexual development (DSD) (Bedrick et al.; Siebert et al.). The care of patients with DSD requires a multidisciplinary expert approach. Pitfalls in diagnosis after donor transplant are highlighted in this Research Topic by Li et. al. (9), who describe the importance of a detailed history and examination in patients presenting with premature ovarian insufficiency and XY karyotype (due to bone marrow transplant from a male sibling donor). Without exploration of this history patients could be misdiagnosed with Swyer syndrome instead of chemotherapy induced premature ovarian insufficiency. These are important clinical lessons for pediatric and adolescent oncology, endocrinology and gynecology clinicians alike, when puberty is delayed after cancer treatment.

There is intense interest in innovative fertility preservation techniques, including fertoprotective agents, which may be used as gonadal protectants during chemotherapy. In this Research Topic, Feng et al. explored if co-administration with melatonin, a free radical scavenger and a broad spectrum antioxidant, could reduce cyclophosphamide-induced primordial follicle loss in mice. The authors demonstrated that co-treatment with melatonin significantly prevented cyclophosphamide-induced apoptosis, of ovarian granulosa cells through inhibition of mitochondrial apoptosis pathways. Anti-mullerian hormone (AMH) expression was maintained, preventing non-growing follicle activation, maintaining ovarian reserve, and increasing litter size, providing new evidence for melatonin as a potential adjuvant chemotherapy agent of the future.

With respect to advances in gonadal tissue preservation, Moussaoiu et al. (10) reported the feasibility and safety of testicular tissue preservation from a Swiss multi-center network, adding to the currently limited body of literature on this technology (Figure 1). This study demonstrated high acceptance rates of testicular tissue preservation by families (90%), despite the experimental nature of the procedure. Importantly, the authors evaluated the quality of gonadal biopsies in a population of whom approximately 50% had received prior moderate gonadotoxic risk therapy (median cyclophosphamide equivalent dose of 5.5 mg/m^2). This has not previously been well reported. Approximately 30% of the study population had a diagnosis of leukemia, where common consensus is to offer fertility preservation as an interval procedure (prior to bone marrow transplant) when minimum residual disease is negative (11).Tumor cells were found in one biopsy (through immunohistochemistry), highlighting the importance of pathological evaluation of all samples and the need for advancements in molecular technologies to detect malignant cells in gonadal tissue prior to transplantation.

An improved understanding of the reproductive capability of collected gonadal tissue was further discussed by Baston-Bust et al., who studied a German cryobank focusing on ovarian tissue cryopreservation. The authors suggested that examination for follicle density be undertaken in order to plan the number of cortex pieces to transplant in the future when parenthood is required. The results of both of these studies highlight the knowledge gaps in pediatric oncofertility, and the importance of oncofertility registries in monitoring efficacy of fertility preservation technologies in the future (12). Further highlights reported on the successful

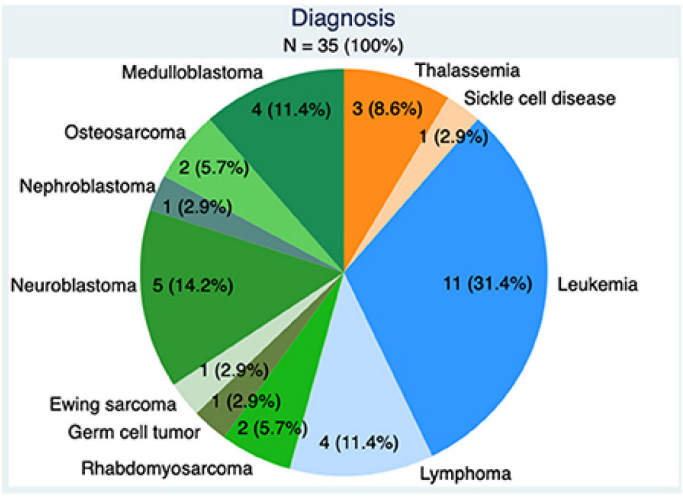

FIGURE 1
Underlying diagnoses requiring gonadotoxic therapy in boys: benign conditions (orange) Hematologic malignancies (blue) and solid tumors (green).

achievements of the Fertiprotekt network of 150 centers, founded in 2006, which have now undertaken over 300 ovarian tissue preservation procedures in German-speaking countries, and counselled 60 patients aged 15 years or less, demonstrating the importance of achieving equitable care and meaningful research outcomes through sustained collaboration.

Finally, a novel study on the potential to harness the power of social media to disseminate knowledge on the impact of cancer treatment on fertility and fertility preservation options explores questions on how best to communicate with digital savvy adolescents and young adults in ways they may find more familiar. (Martinez-Ibarra et al.).

Author contributions

All authors contributed to conception, critically evaluating the drafts and approving the final draft.

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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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CASE REPORT
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Case Report: Is It Premature Ovarian Insufficiency or Swyer Syndrome After Bone Marrow Transplantation?

Hui Li[1], Jin Li[1], Xiaohong Li[1], Hong Yi[1], Qixiu Ren[1] and Xiaoyan Chen[2,3]**

[1] Department of Reproductive Health, Shenzhen Baoan Women's and Children's Hospital, Shenzhen University, Shenzhen, China, [2] Department of Obstetrics and Gynaecology, Shenzhen Baoan Women's and Children's Hospital, Shenzhen University, Shenzhen, China, [3] Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Hong Kong, Hong Kong SAR, China

Introduction: Iatrogenic factor is one of the recognized causes for premature ovarian insufficiency. The aim of this case report was to present a rare case with premature ovarian insufficiency and 46, XY karyotype after bone marrow transplant (BMT) for thalassaemia major at childhood. We also reviewed some relevant literature in this report.

Case Presentation: A 17-year-old girl was presented with primary amenorrhea and premature ovarian insufficiency after receiving chemotherapy and BMT from her brother due to thalassaemia major at childhood. She had poor secondary sex characteristics, assessed as stage I for the development of breasts and external genitalia based on the Tanner scale. Transabdominal ultrasound showed small uterus with visible endometrial lining and small ovaries. Laboratory data showed hypergonadotropic hypogonadism profile with low level of estrogen and high level of follicular-stimulating hormone (FSH). Patient's peripheral lymphocytes karyotype was 46, XY.

Conclusions: This case was diagnosed as a chemotherapy induced premature ovarian insufficiency. Patient's peripheral lymphocytes karyotype (46, XY) after she received BMT from a male donor was a misleading finding, and the case could be easily misdiagnosed as Swyer syndrome. A correct diagnosis in such cases should depend not only on the recent clinical findings, but also on the detailed medical history. To prevent premature ovarian insufficiency in similar cases, fertility preservation should be offered to girls before they receive chemotherapy, total body irradiation and BMT.

Keywords: premature ovarian insufficiency (POI), bone marrow transplantation, thalassemia major, primary amenorrhea, 46, XY karyotype

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Mahmoud Salama, Michigan State University, United States

Reviewed by:
Nalini Mahajan, Independent Researcher, New Delhi, India
Senay Savas Erdeve, Dr Sami Ulus Child Health and Diseases Training and Research Hospital, Turkey

***Correspondence:**
Jin Li
jennypodli@163.com
Xiaoyan Chen
chenxiaoyan1214@yahoo.com

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INTRODUCTION

Premature ovarian insufficiency affects around 1% of women under the age of 40. It usually presents as oligo- or amenorrhea for at least 4 months and FSH level over 40 IU/ml, checked twice at least 4 weeks apart (1, 2). When serious ovarian function depletion occurs in childhood, most patients will experience primary amenorrhea.

Iatrogenic factors are one of the recognized causes for premature ovarian insufficiency, including radiation treatment andchemotherapy. The use of chemotherapy, radiotherapy and bone marrow transplantation (BMT) to treat malignant and nonmalignant diseases in girls and young women

has become more common (3). Therefore, the awareness of fertility preservation for this group of subjects should be emphasized since the late side effects of BMT, though usually not life threatening, may significantly impair quality of life in adults (3), in whom gonadal failure is a common long-term endocrine consequence of BMT.

In this case report, we present a patient with primary amenorrhea and premature ovarian insufficiency after chemotherapy and BMT due to thalassaemia major, whose peripheral lymphocytes karyotype result (46, XY) could lead to a misdiagnosis of disorders of sexual differentiation.

CASE PRESENTATION

This report was approved by the local hospital Ethics Committee. Patient was a 17-year-old girl who attended our outpatient clinic for evaluation of primary amenorrhea. Her past medical history revealed short period of chemotherapy followed by BMT therapy due to thalassaemia major at the age of eight. She reported to be free of the disease currently and did not take any medication. As an adolescent, the patient came with her mother, reported a lack of menstrual flow and was presented with poor secondary sex characteristics.

During the visits, she was assessed as stage I for the development of breasts and external genitalia according to the Tanner scale. The presence of a vaginal opening was found by gynecological examination. Transabdominal ultrasound showed small uterus (2.1 × 1.5 × 1.0 cm) with visible endometrial lining; both ovaries were measured as 1.6 × 0.8 cm (**Figure 1**). Laboratory data showed low concentration of estradiol (<55 pmol/l) and significantly elevated levels of serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (125.93 and 31.59 mIU/ml, respectively). Anti-Mullerian hormone (AMH) concentration was extremely low (0.04 ng/ml). The remainder of the results, including levels of basal thyroid hormones, TSH, cortisol, 17-hydroxyprogesterone and prolactin, were within the reference range. X-ray of the left wrist showed that the bone age was 13 years old (**Figure 2**). Patient's peripheral lymphocytes karyotype was 46, XY. A more detailed history was then taken and it was found that the patient had BMT from her brother. Based on the above characteristics, the patient was diagnosed as primary amenorrhea and premature ovarian insufficiency. A more detailed history was then taken and the entire medical record was checked carefully. When the patient was 8 years old, she had HLA-matched bone marrow hematopoietic stem cell from her brother. Before BMT, she had bone marrow aspiration and bone marrow cell karyotype was shown as 46, XX. However, when the patient came to our clinic, she refused to reassess the karyotype using other different cells, such as skin cells. Based on the above characteristics, the patient was diagnosed as primary amenorrhea and premature ovarian insufficiency.

Currently, the patient receives low dose of estrogen therapy (0.25 mg estradiol valerate orally daily) to allow the growth and development of breast and reproductive organs as well as the skeleton. The patient will be followed-up every 3 months for regular checkups in our outpatient clinic. When there is improvement of the uterus size and endometrial thickness, hormone replacement treatment using estradiol and norgestrel may be given to induce menstruation.

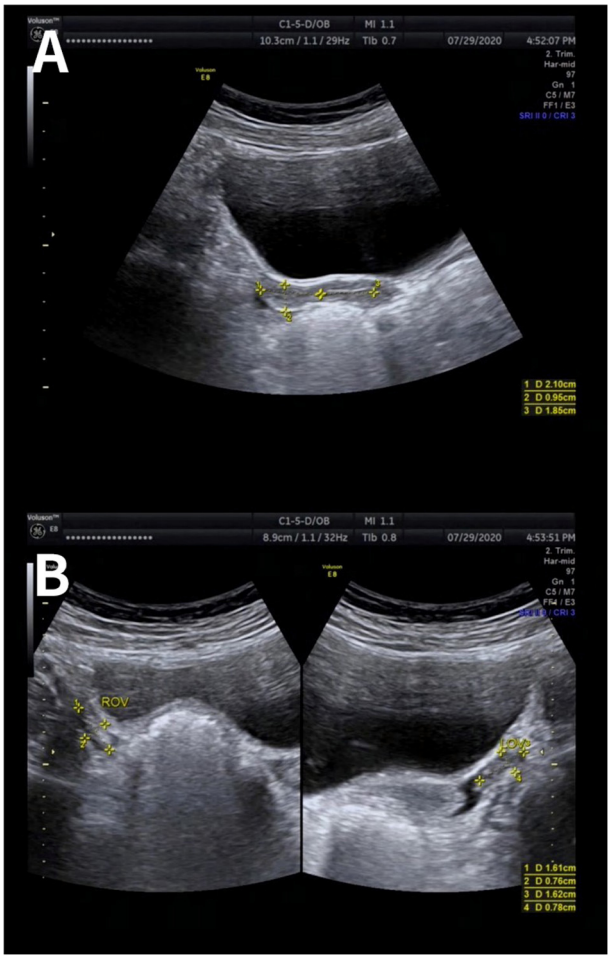

FIGURE 1 | Ultrasonography image of uterus and ovaries. Transabdominal ultrasound showed small uterus (2.1 × 1.5 × 1.0 cm) with visible endometrial lining **(A)**. Both ovaries were measured as 1.6 × 0.8 cm **(B)**.

DISCUSSION AND CONCLUSION

In females, menarche is the most significant corporal change during adolescence. Primary amenorrhea is defined as the absence of menstruation by the age of 15 with developed secondary sexual characteristics or 3 years after thelarche (4). There are several classifications of causes of primary amenorrhea, including anatomic defects, primary hypogonadism, hypothalamic causes, pituitary causes, chromosomal abnormalities and other endocrine gland disorders (5).

The prevalence of 46, XY karyotype in females with primary amenorrhea, either with pure gonadal dysgenesis or complete androgen insensitivity syndrome, is around 3–6% (6). Pure

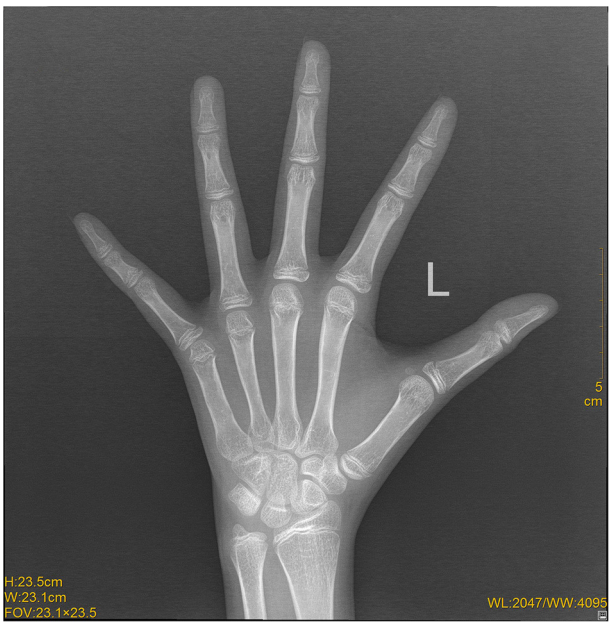

FIGURE 2 | X-ray image of the left wrist. X-ray showed that the bone age was 13 years old.

gonadal dysgenesis, also known as Swyer syndrome, may be recognized when there are not normally differentiated testes despite male karyotype. It has been reported that the mutation in several genes is responsible for Swyer syndrome, among which SRY appears to be the most significant one and responsible for 10–20% of cases (7). Patients with Swyer syndrome are typically tall-statured and with female genitalia. Another feature is hypergonadotropic hypogonadism with low levels of estrogen and AMH and high level of FSH, which results in poor breast development, amenorrhea, increased risk of cardiovascular disease (7). With regards to complete androgen insensitivity syndrome, tissues are not reactive to testosterone due to the mutation in gene encoding androgen receptor, although testes are developed and their hormone secretion function is preserved. Affected individuals are with the external female phenotype and genitalia due to the normal conversion of excessive testosterone to estrogen. However, Mullerian derivatives are not preserved and testes may be situated either in the abdominal cavity or throughout the inguinal canal or in the labia majora. Hormonal profiles usually show elevated testosterone and detectable estrogen concentrations (8).

The clinical features in our patient presented in this report, including female external genitalia, presence of uterus, hypergonadotropic hypogonadism, low AMH level and 46, XY karyotype, can be easily misdiagnosed as Swyer syndrome. However, when digging the past history of the patient, it was found that the appearance of 46, XY karyotype in the peripheral blood in this patient is due to the BMT from a male donor. In addition, her karyotype was 46, XX in the bone marrow cells before BMT. This clue can help to rule out the diagnosis of Swyer syndrome. There are two similar cases reported previously. One study reported a childhood cancer survivor with premature ovarian insufficiency and 46, XY karyotype in lymphocytes after chemotherapy and BMT from an unrelated male donor, and the karyotype appeared to be 46, XX in the swab sample from the cheek, which contained fibroblasts (9). The other report showed a patient with primary amenorrhea and 46, XY karyotype after receiving BMT from her brother and chemotherapy due to acute myeloid leukemia (10). The authors from both reports also found a high likelihood of misdiagnosis of Swyer syndrome, which was similar to our report. Therefore, the comprehensive collection of detailed present and past medical history is important for a correct diagnosis. Moreover, although the patient in this report refused to check karyotype in other different cells other than peripheral lymphocytes, it is still strongly recommended using other cells (such as skin cells) to reassess karyotype after BMI in similar cases to confirm the diagnosis.

In this case presented, several possible causes may be contributing to the observed iatrogenic premature ovarian insufficiency. After repeated red blood cell transfusions, patients with thalassemia major may have ovarian impairment due to iron overload, when the transferrin-dependent system is inhibited through ferritin saturation pathway and excessive iron accumulation occurs through the non-transferrin bound iron (NTBI) pathway (11). Although the most common endocrinopathy in patients with thalassemia is the hypogonadism resulting from iron deposition in the hypothalamic and/or pituitary cells (12), it is also worth noting that iron overload may affect ovarian function directly as well. An earlier study has shown an inverse correlation between AMH level and NTBI (13), suggesting suspected ovarian tissue iron overload in women with thalassemia major. Another study also demonstrated that AMH level and antral follicle count are significantly decreased in women with transfusion-dependent thalassemia major compared with age-matched controls (12). These findings support a deleterious effect of iron overload on ovarian tissue, which may result in an increase in reactive oxygen species and the subsequent acceleration in follicular aging (14). An earlier study has found high redox activity in the ovarian follicular fluid from a woman with thalassemia major, which suggested that redox-active iron ions may mediate free radical production and induce ovarian tissue injury (15). Therefore, there is a need to better define the appropriate chelation regimens and antioxidant supplementations regarding reproductive function in women with thalassemia major receiving blood transfusion treatment. However, whether ovarian function is impaired by a direct effect of iron overload is not clearly understood yet.

Another important factor responsible for the ovarian impairment in this case may be BMT, including chemotherapy and/or total body irradiation (TBI). The extent of follicular impairment can be affected by the type of TBI protocol as well as the age of the patient receiving BMT. In terms of chemotherapy, busulfan appears to be the most gonadotoxic regimen, with reported prevalence of premature ovarian failure as high as 100% (16, 17). Low-dose cyclophosphamide (200 mg/kg) is considered to be much less gonadotoxic compared with other regimens, with

good recovery rates of clinical ovarian function, particularly in women younger than 25 years old (18, 19). In contrast, TBI appears to be a more toxic treatment prior to BMT and most patients undergoing TBI experience gonadal failure. Compared with TBI administration after puberty, TBI administered prior to puberty is reported to be less gonadotoxic and around 40–60% of patients experienced spontaneous puberty (20, 21). This might be due to the higher number of non-growing follicles found in younger girls and some other particular anatomical or paracrine factors, which might lead to a higher resistance to fibrosis implicated in the mechanisms of ovarian damage (22).

Therefore, fertility preservation is crucial for this cohort of patients, particularly prior to repeated blood transfusion treatment and BMT. In post-pubertal women, fertility preservation methods consist of oocyte freezing, embryo freezing, ovarian tissue cryopreservation and GnRH agonist application (23), while in pre-pubertal girls, ovarian tissue cryopreservation is the main option, although ovarian shielding from radiotherapy may also be available considerations (24). Ovarian cortical tissue, which contains a large reserve of oocytes in the primordial follicles, can be frozen, stored and then re-implanted after BMT treatment and pregnancy can be then possibly achieved, either naturally or by assisted reproductive technique (25). Whereas, restoration of fertility is the primary indication for re-implantation of ovarian tissue, the ovary is also an endocrine organ and restoration of hormonal function may also be an indication (26). However, ovarian tissue cryopreservation is an invasive and still experimental procedure for young girls, which requires laparoscopic surgery. Hence, more long-term follow-up data will be needed to verify the effect of ovarian tissue cryopreservation in this cohort of patients.

To conclude, this is a case report of a patient presenting with primary amenorrhea, premature ovarian insufficiency and 46, XY karyotype in peripheral blood after receiving chemotherapy and BMT from her brother in childhood due to thalassemia major, which can be easily misdiagnosed as Swyer syndrome. It is important that gynecologists be aware that not only the clinical manifestations, but also the detailed medical history, are crucial for a correct diagnosis and the following treatment. In light of the relatively high prevalence of thalassemia, particularly in the southern China, fertility preservation should be considered for young girls who are going to have chemotherapy, total body irradiation and BMT.

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 the Institutional Review Board (IRB) of Shenzhen Baoan Woman's and Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HL, JL, and XC: substantial contribution to the conception and design of the work and manuscript drafting. HL, XL, HY, and QR: performed investigations on the patient. HL and XC: participation in acquisition of the literature. All authors have read and approved the final article.

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Fertility Preservation for Adolescent and Young Adult Transmen: A Case Series and Insights on Oocyte Cryopreservation

Francesca Barrett[1], *Jacquelyn Shaw*[2], *Jennifer K. Blakemore*[2] *and Mary Elizabeth Fino*[2*]

[1] *Department of Obstetrics and Gynecology, New York University Grossman School of Medicine, New York, NY, United States,* [2] *Department of Reproductive Endocrinology and Infertility, New York University Langone Fertility Center, New York, NY, United States*

Background: The opportunity for fertility preservation in adolescent and young adult (AYA) transmen is growing. Many AYA transmen desire future biologic children and are interested in ways to preserve fertility through oocyte cryopreservation prior to full gender affirmation, yet utilization of oocyte cryopreservation remains low. Additionally, standard practice guidelines currently do not exist for the provision of oocyte cryopreservation to AYA transmen. Our objective was to review our experience with oocyte cryopreservation in adolescent and young adult transmen in order to synthesize lessons regarding referral patterns, utilization, and oocyte cryopreservation outcomes as well as best practices to establish treatment guidance.

Methods: This is a case series of all AYA transmen (aged 10 to 25 years) who contacted, consulted or underwent oocyte cryopreservation at a single high volume New York City based academic fertility center between 2009 and 2021.

Results: Forty-four adolescent and young adult transmen made contact to the fertility center over the study period. Eighty percent (35/44) had a consultation with a Reproductive and Endocrinology specialist, with a median age of 16 years (range 10 to 24 years) at consultation. The majority were testosterone-naive (71%, 25/35), and had not pursued gender affirming surgery (86%, 30/35). Expedited initiation of testosterone remained the most commonly cited goal (86%, 30/35). Fifty-seven percent (20/35) pursued oocyte cryopreservation. Ninety-five percent (19/20) underwent successful transvaginal oocyte aspiration, with a median of 22 oocytes retrieved and 15 mature oocytes cryopreserved. There were no significant adverse events. At time of review, no patient has returned to utilize their cryopreserved oocytes.

Conclusions: Oocyte cryopreservation is a safe fertility preservation option in AYA transmen and is an important aspect of providing comprehensive transgender care. Insights from referral patterns, utilization, and oocyte cryopreservation outcomes from a single center's experience with adolescent and young adult transmen can be integrated to identify lessons learned with the goal of providing transparency surrounding the oocyte

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****Correspondence:***
Mary Elizabeth Fino
Mary.Fino@nyulangone.org

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cryopreservation process, improving the education and comfort of patients and providers with fertility preservation, and easing the decision to pursue an oocyte cryopreservation cycle in parallel to gender-affirmatory care.

Keywords: transgender, adolescent reproductive health, oocyte cryopreservation, adolescent and young adult (AYA), fertility, fertility preservation

INTRODUCTION

The prevalence of transgender and gender-diverse adults has doubled over the past decade, with prevalence of AYA transgender youth estimated to be between 0.6% to 3.2% of the U.S population (1–4). Adolescents identify as transgender more often than adults, and gender affirming treatments are occurring at younger ages, often as AYA (ages 10 to 25 years) (1–4). National and international organizations recommend fertility preservation counseling regarding oocyte cryopreservation, embryo banking, and ovarian tissue cryopreservation for transgender male individuals prior to initiating gender-affirming treatments (5–7). Many AYA transmen desire future biologic children and are interested in ways to preserve their fertility (8–14). Fertility preservation has primarily been through oocyte cryopreservation, which is a safe and feasible option that does not require a partner or use of donor sperm (9–14). Despite increasing prevalence, societal support, and interest in fertility preservation, utilization of oocyte cryopreservation remains low in AYA transmen, with several small studies reporting rates between 0% to 7.8% (15–19).

Gaps in utilization of fertility preservation for AYA transmen are multifactorial (20, 21). Some AYA patients prioritize their desire to initiate testosterone as soon as possible and thereby forego fertility preservation and its associated counseling (15, 16, 22). Others defer consultation due to fear surrounding the invasiveness of fertility preservation procedures or potential gender dysphoric triggers (9, 15, 16, 21, 23). There are financial barriers due to the high cost of services often uncovered by insurance as well as systems barriers in the form of primary care providers who are inexperienced with AYA transmen's fertility needs or have difficulty counseling patients based on current fertility preservation outcomes research (9, 15, 16, 23–25). Others consider parenthood with biologic children a low priority during their adolescence (21).

Furthermore, there are no standard practice guidelines for the provision of oocyte cryopreservation to AYA transmen (6). Lack of standardized fertility preservation practices for AYA transmen reinforces these gaps by reducing transparency surrounding fertility preservation and precluding patient and provider education (22). As a result of low utilization, research on the experiences and outcomes of oocyte cryopreservation within AYA transmale population is limited. Current data are primarily from case reports and small case series reporting general findings surrounding gonadotropin requirements, hormonal levels during stimulation, and mature oocyte retrieval yields (10, 26–31).

Therefore, we performed a case series of AYA transmen interested in fertility preservation to evaluate referral patterns, utilization, and oocyte cryopreservation outcomes to identify important care points. Our objective was to synthesize experiences across patients who contacted, consulted, and underwent oocyte cryopreservation to define lessons learned and best practices for fertility preservation amongst AYA transmen.

MATERIALS AND METHODS

Design

A case series was performed between October 2009 and June 2021 of all AYA transmen who made contact with New York University Langone Fertility Center (NYULFC), a high volume New York City based academic fertility center capable of oocyte cryopreservation and embryo banking for fertility preservation. This study was performed with New York University Grossman School of Medicine Institutional Review Board approval (i13-00389).

Subjects

All AYA subjects, defined as individuals between the ages of 10 and 25 years old, who contacted NYULFC during the study period were reviewed. Patients were included if documentation confirmed they identified as transmale or gender non-conforming individuals assigned female at birth. Patients were excluded if 1) they identified as transfemale or gender non-conforming individuals assigned male at birth, or 2) they were younger than 10 or older than 25 years at time of initial consultation.

Variables and Data Collection

Electronic medical records were reviewed to extract all demographics and outcome variables. All records were reviewed for referral source (provider and institution) and age at contact. Records of AYA transmen who proceeded with consultation with a Reproductive Endocrinology and Infertility (REI) physician were reviewed for age at consultation, age at initiation of gender affirmation through reported through pronoun changes, breast binders, or other social changes, pre-consultation gender affirming treatments including menstrual suppression, testosterone, gender affirming surgery, and hormonal implants, post-consultation gender affirming treatments, and goals at time of consultation. Records of AYA transmen who proceeded with fertility preservation were reviewed for assisted reproductive technology outcomes, including baseline hormonal labs, ultrasound modality for monitoring, stimulation dosing, trigger dosing, days of stimulation, number of oocytes retrieved, number of mature and immature oocytes frozen, total number of cycles, oocyte disposition, adverse events, and documentation of practices to

minimize dysphoria. Episodes of gender dysphoria were assessed by reviewing documentation of pre/post procedure progress notes described by REIs, social workers, and/or primary care providers. Records of AYA patients who did not proceed with fertility preservation were reviewed for post-contact/consultation gender affirming treatments and reported reasons for deferral that were documented within the medical chart to REIs, social workers, and/or primary care providers following consultation. The primary outcome was number of mature oocytes cryopreserved. Secondary outcomes included number of immature oocytes cryopreserved, utilization of consultation and fertility preservation, rates of reported cycle-related gender dysphoria, initiation of gender affirming treatments following contact, consultation, and fertility preservation, rates of low trigger response and cancelled cycles, and rates of adverse outcomes.

Ovarian Stimulation and Oocyte Cryopreservation

Two protocols were utilized for controlled ovarian hyperstimulation: a gonadotropin-releasing hormone antagonist protocol and a low-dose down-regulation protocol with leuprolide acetate. Protocols were prescribed to each subject per provider discretion. Subjects received gonadotropins (recombinant follicle-stimulating hormone, human menopausal gonadotropins, or both) for all protocols, with follicular growth monitored by serial serum estradiol levels and ultrasound monitoring. For antagonist protocols, the gonadotropin-releasing hormone antagonist was initiated when 1) a lead follicle was identified as 13 mm^2; 2) the estradiol was >1,000 pg/mL; or 3) at the discretion of the primary provider. Either human chorionic gonadotropin (hCG) alone or with leuprolide acetate, as appropriate, was used for the trigger of final oocyte maturation. Oocyte aspiration was scheduled for 35 hours after trigger administration as is routine/standard at our center. Retrievals were performed *via* ultrasound-guided transvaginal aspiration. Oocytes were cryopreserved *via* slow frozen methodology or *via* vitrification, as was standard of care in the embryology laboratory at the time of cryopreservation using previously described techniques (32).

Analysis

Continuous variables were assessed for normality using Kolmogorov-Smirnoff test and determined to be non-parametric; thus Mann-Whitney tests were used to compare continuous variables. Categorical variables were analyzed using the Chi-squared tests or Fisher's exact, where appropriate, to assess for group differences in survey measures by demographic and professional characteristics. An alpha error of 0.05 was considered statistically significant. Descriptive results are reported as percent, counts, median, and range. Key themes and lessons were identified for areas of excellences and gaps.

RESULTS

Referrals and Utilization

A total of 44 AYA transmen contacted the NYULFC between October 2009 and June 2021 with a median age of 17 years (range 10-24 years) at time of contact. Most referrals came from providers within the same institution (77%, 34/44), with two providers (a pediatric adolescent physician and a psychologist) from the Gender and Sexuality (G&S) service at the NYU Hassenfeld Children's Hospital referring the majority of patients (**Figure 1A**). Other referring providers included in-institution psychiatrists/psychologists, an urologist, an endocrinologist, a pediatrician, and a plastic surgeon and a gynecologist that both perform gender affirming surgery. Only one patient was referred from an outside institution (**Figure 1A**).

Eighty percent (35/44) of patients who contacted the fertility center proceeded with consultation with a REI physician (**Figure 1B**). Nine percent (3/35) of consultations occurred before 2017, with 69% (24/35) of consultations between 2019-2021. Ninety-four percent (33/35) of patients who consulted with

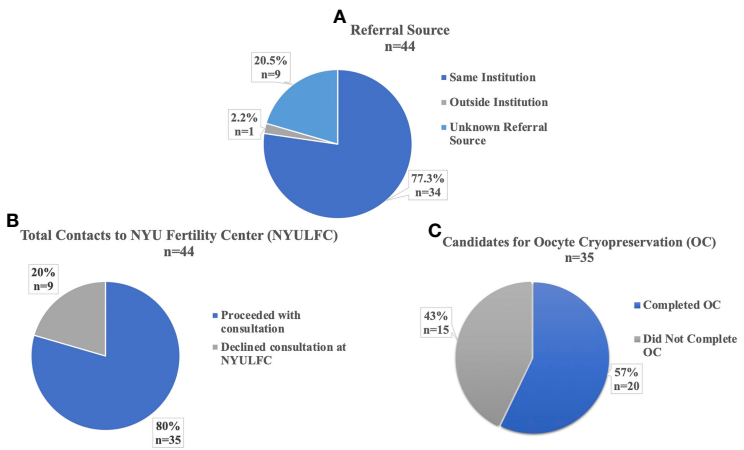

FIGURE 1 | Referrals and Utilization of AYA transmen at NYU Langone Fertility Center. **(A)** Institutional Source for Referral to NYU Fertility Center. **(B)** Percent of Total Contacts to NYU Fertility Center who Proceeded with Consultation. **(C)** Percent of Consultations who were Candidates for Oocyte Cryopreservation.

a REI were eligible for oocyte cryopreservation (OC). The two ineligible patients were aged 10 and 13 years and were Tanner stage II; both were encouraged to return at later development stages for OC or consider tissue cryopreservation at another institution, as ovarian tissue cryopreservation was not offered at our center in the study period. Fifty-seven percent (20/35) of consulting patients pursued OC (**Figure 1C**). No patients pursued embryo cryopreservation. Patients proceeded quickly with OC following consultation, with 73% (16/22) of cycles starting within two months of consultation, with median one month (range 1-17 months) between consultation and cycle start date. Median age at consultation was 16 years (range 10-24 years) and median age at cycle start was 16 years (range 12-25 years).

Records were available for all patients who deferred consultation with a REI physician, though documentation of reason for deferral was limited (**Table 1**). Most patients that deferred consultation were on testosterone before or initiated shortly after initial contact to the fertility center with all reporting awareness of the possible fertility impact. Five patients expressed certainty that they were not interested in genetic children. One patient underwent oocyte cryopreservation at a different fertility center and subsequently started testosterone. Another patient was concerned fertility preservation would be too distressing and opted to begin pubertal suppression with leuprolide acetate.

Consultation Characteristics and Goals

General characteristics for those who sought consultation can be seen in **Table 2**. At time of consultation, patients were at varying stages of their gender affirmation. Most patients were earlier in their gender affirmation, as the majority were testosterone-naive (71%, 25/35), and had not pursued gender affirming surgery (86%, 30/35). Patients reported differing primary goals for their consultation including fertility preservation prior to testosterone or gender affirming surgery (13/35), expedited initiation of testosterone (9/35), initiation of testosterone alongside other expedited gender affirming treatments (8/35), family building (2/35), menstrual suppression (1/35), or not documented (2/35). Only seven patients expressed certainty of their desire for a genetic child, though many were concerned that they might miss the opportunity to preserve fertility prior to starting definitive treatments. Several patients' goals for affirmation outweighed their goals for fertility preservation. Of the patients who did not seek oocyte cryopreservation following consultation, four patients underwent gender affirming top surgery, one patient underwent gender affirming bottom surgery, and four patients began testosterone though reported desire to complete fertility preservation in the future. In comparison, several patients who prioritized fertility preservation pursued gender affirming treatments after completing oocyte cryopreservation, including twelve testosterone naïve patients initiating testosterone and nine patients undergoing their first gender affirming surgery.

Dysphoria Protection Protocols

Care was taken to minimize gender dysphoric events during consultation, ovarian stimulation, and oocyte retrieval, in accordance with World Professional Association for Transgender Health (WPATH) "Standards of Care for hormone-prescribing physicians for the Health of Transsexual, Transgender, and Gender Nonconforming People," which outlines the specific responsibilities of hormone-prescribing physicians (5). At the initial consultation, alongside pertinent discussion of patient's affirmation goals, health history, physical exam, and relevant laboratory tests, patients were asked preferred language for first name, pronouns, as well as possible triggering gendered terms, including but not limited to menstruation, female organs, and oocytes/eggs. No patient underwent pelvic examination at initial consultation per patient preference.

During ovarian stimulation, patient's medical records had name alerts to ensure patients were called by appropriate preferred names and pronouns. Ovarian stimulation ultrasound monitoring was completed transabdominally for 82% (18/22) of cycles. Of the remaining four cycles, transvaginal ultrasound was utilized to assess ovarian pathology at initial scan (2/22), to visualize ovaries that were unable to be seen abdominally (1/22), or to accommodate patient

TABLE 1 | Characteristics of AYA transmen who did not seek consultation.

Age Range at Contact (years)	Initiation of Testosterone prior to consultation	Reason for declining consultation	Clinical Next Steps
16-18	No	Not interested in biologic offspring	Began Testosterone Oocyte cryopreservation at alternative institution Began testosterone
16-18	No	Oocyte cryopreservation at different institution	Underwent gender affirming top surgery Began leuprolide acetate
13-15	No	Concern for dysphoria with fertility preservation	Underwent gender affirming top surgery
19 and older	Yes	Not interested in biologic offspring	Underwent gender affirming bottom surgery and phalloplasty
19 and older	Yes	Not interested in biologic offspring	Continued testosterone
19 and older	No	Not interested in biologic offspring and desire to initiate testosterone	Began testosterone
19 and older	Yes	Not indicated	Continued testosterone
16-18	No	Desire to initiate testosterone	Began testosterone
13-15	Yes	Not interested in biologic offspring	Continued testosterone

TABLE 2 | Characteristics of AYA transmen who underwent fertility preservation consultation with a Reproductive Endocrinology and Infertility physician.

	Declined Fertility Preservation n = 15	Underwent Oocyte Cryopreservation n = 20	P value
General Characteristics of Referrals			
Age at consultation [years, median (range)]	18 (10-24)	16 (12-23)	0.16
Age at gender identity affirmation [years, median (range)]	12 (9-17)	14 (11-21)	0.56
Time between Gender Affirmation and Consult [years, median (range)]	2 (1-13)	2 (1-4)	0.56
Pre-Consultation Gender Affirming Treatments			
Menstrual suppression with oral contraception	27% (4/15)	15% (3/20)	0.31
Pubertal blocker with leuprolide acetate	20% (3/15)	10% (2/20)	0.63
Testosterone	33% (3/15)	10% (2/20)	0.63
Gender Affirmatory Top Surgery	27% (4/15)	5% (1/20)	0.14
Post-Consultation Gender Affirming Treatments			
Menstrual suppression with oral contraception	27% (4/15)	15% (3/20)	0.31
Menstrual suppression with leuprolide acetate	27% (4/15)	10% (2/20)	0.39
GNRH implant	0% (0/15)	5% (1/20)	1.00
Testosterone	47% (7/15)	70% (14/20)	0.69
Gender Affirmatory Top Surgery	53% (8/15)	45% (9/20)	0.28
Gender Affirmatory Bottom Surgery	7% (1/15)	5% (1/20)	1.00
Fertility Goals			
Unsure of desire of future biological children (%)	53% (8/15)	55% (11/20)	0.67
Certain of desire of future biological children (%)	13% (2/15)	25% (5/20)	
No documentation of desire of future biologic children (%)	33% (5/15)	20% (4/20)	

preference for vaginal monitoring (1/22). No patients requiring transvaginal monitoring had been on testosterone and no dysphoric events were recorded related to transvaginal monitoring. There were no documented events of patients experiencing gender dysphoria specifically related to ovarian stimulation.

For oocyte retrieval procedures, patients were allowed to maintain chest binder and wear undergarments in the operating room until fully sedated if so desired. Patients were positioned into dorsal lithotomy only after deep sedation was achieved. All oocyte aspirations were completed transvaginally. Patients were counseled preoperatively on possibility of hymenectomy at time of retrieval to accommodate the transvaginal ultrasound probe, but no hymenectomies were required. Undergarments were replaced prior to patient's movement to the recovery area. Special accommodations were encouraged during the recovery period, such as private recovery area or inclusion of parents if desired during recovery. There were no documented events of patients experiencing gender dysphoria during the oocyte retrieval or in the postoperative period.

Fertility Preservation Outcomes

Individual outcomes for oocyte cryopreservation can be seen in **Table 3A** and cumulative summary characteristics in **Table 3B**. There were a total of 20 patients who attempted oocyte cryopreservation, 19 patients who underwent oocyte retrieval, and two patients who completed two cycles of oocyte cryopreservation, for a sum total of 21 completed oocyte cryopreservation cycles and one cancelled cycle. Median age at cycle start was 16 years (range 12-25 years). Baseline anti-mullerian hormone (AMH) level was median 3.26 ng/ml (range 0.44-12.87 ng/ml). 95% (21/22) of cycles had unmedicated cycle day two starts. Median cycle day two follicle stimulating hormone (FSH) was 5.65 mIU/mL (range 1.7-9.5 mIU/mL) and cycle day two estradiol (E2) was 43 pg/mL (range 19-163 pg/mL). There was one random start cycle to expedite oocyte cryopreservation and no luteal start cycles. Ninety-five percent (21/22) of cycles were gonadotropin-releasing hormone antagonist protocols. Notably, one patient with a baseline luteinizing hormone (LH) level of 0.4 mIU/L, had serial LH level monitoring but never required administration of GnRH antagonist prior to retrieval. The remaining cycle utilized a low-dose down-regulation protocol with leuprolide acetate prior to administration of gonadotropins. Notably, no cycles included letrozole in the protocol. Median total gonadotropin dose was 2375 IU (range 825-8075 IU) over median 10 days of stimulation (range 8-21 days), with median initial dosages 150 IU FSH (range 50-300 IU) and 75 IU human menopausal gonadotrophin (HMG) (range 0-150 IU).

Eighty-six percent of cycles (19/22 cycles) were triggered with a combination of recombinant human chorionic gonadotropin (hCG, 1000U, 5000U, or 10,000U) and leuprolide acetate (single 40U dose or two 40U doses 12 hours apart). Five patients had a low response to the combination trigger (LH < 40mIUm/mL) with one patient (LH level 0.9 mIU/mL and HCG level 21mIU/mL), requiring an additional hCG boost of 5000U while maintaining standard 35 hour post initial trigger retrieval time.

Two patients were on testosterone prior to initiation of cycle and resumed testosterone following OC (**Table 3A**). Both were weaned off of testosterone prior to cycle start date, one patient for three months and the other for two months. One patient with polycystic ovarian syndrome was cancelled for low response with a maximum estradiol 943 pg/mL and two dominant follicles after 21 days of stimulation. This patient did not proceed with an additional oocyte cryopreservation cycle following cancellation, but proceeded to initiation of testosterone.

A median of 22 oocytes (range 5-59) were retrieved. 67% of cycles (14/21) had at least 20 oocytes retrieved and cryopreserved.

TABLE 3A | Oocyte Cryopreservation Outcomes for Individual AYA Transmale Cycles.

Age Range at gender affirmation (years)	Age Range at cycle start (years)	Pre-consultation gender affirming therapy	AMH (ng/ml)	Protocol	Total gonado-trophin dose (IU)	Duration of stimulation (days)	E2 max (pg/mL)	Trigger	Total oocytes retrieved	MII oocytes frozen	MI oocytes frozen	GV oocytes frozen
12 and under	12 and under		1.58	Antagonist	4050	10	4212	1000U hCG + double GnRH-a, 5000U hCG boost	13	3	8	1
				Antagonist	5400	13	5175	10,000U hCG + double GnRH-a	9	6	1	2
13-15	13-15	Leuprolide acetate	1.53	Antagonist	3700	11	4288	1000U hCG + double GnRH-a	26	5	0	20
13-15	13-15		3.10	Antagonist	1750	9	3049	1000U hCG + single GnRH-a	15	9	3	2
13-15	13-15		3.26	Antagonist	2350	9	3822	1000U hCG + double GnRH-a	22	16	1	4
ND	13-15		0.73	Antagonist	1850	9	1059	1000U hCG + double GnRH-a	5	5	0	0
				Antagonist	2825	9	3084	1000U hCG + double GnRH-a	15	8	6	1
13-15	13-15	Oral contraception	2.70	Antagonist	2100	9	4023	1000U hCG + double GnRH-a	25	20	0	5
13-15	13-15		4.64	Antagonist	3250	13	2045	1000U hCG + single GnRH-a	19	14	2	3
ND	13-15		3.27	Antagonist	2775	10	2344	5000U hCG + double GnRH-a	22	15	3	4
13-15	16-18		9.84	Antagonist	825	8	5040	1000U hCG + double GnRH-a	32	18	3	10
13-15	16-18		3.00	Antagonist	1725	8	2848	1000U hCG + double GnRH-a	26	19	0	6
13-15	16-18	Oral contraception	3.60	Antagonist	2400	9	4393	1000U hCG + double GnRH-a	22	18	0	4
13-15	16-18		12.87	Antagonist	1925	10	6091**	1000U hCG + double GnRH-a	43	14	3	20
13-15	16-18	Oral contraception	0.44	Antagonist	4500	11	2673	1000U hCG + double GnRH-a	9	8	1	0
ND	16-18		5.93	Antagonist	2325	12	3151	10,000U hCG	21	17	0	0
ND	16-18		0.59	Antagonist	4050	10	3288	1000U hCG + double GnRH-a	33	21	8	3
ND	16-18		3.27	Antagonist	1750	8	2864	1000U hCG + single GnRH-a	30	26	1	3
13-15	16-18		5.77	Antagonist	8075	21	943^	–	–	–	–	–
16-18	19 and older		6.18	Antagonist	2550	10	4007**	1000U hCG + double GnRH-a	26	23	2	1
ND	19 and older	Testosterone + Leuprolide acetate	ND	Low-dose leuprolide acetate downregulation	1900	11	6969**	10,000U hCG	59	35	9	0
19 and older	19 and older	Testosterone	7.35	Antagonist	2175	11	2792**	1000U hCG + double GnRH-a	20	18	1	1

ND, not documented.
AMH, Anti-mullerian hormone.
hCG, human chorionic gonadotropin.
E2, Estradiol level.
Total oocytes, Total oocytes (MII, MI, GV).
MII, Metaphase II oocytes.
MI, Metaphase I oocytes.
GV, Germinal Vesicle oocytes.
GnRH-a, gonadotrophin releasing hormone agonist,
Antagonist, gonadotropin-releasing hormone antagonist protocol.
Low-dose leuprolide acetate downregulation = provera for 10 days, leuprolide acetate 10U starting day 6 of provera reducing to 5U on cycle day 2 with initiating of gonadotrophins.
***Denotes experienced ovarian hyperstimulation syndrome (OHSS).*
^Denotes canceled cycle.

TABLE 3B | Oocyte Cryopreservation Outcomes for All AYA Transmale Cycles.

Baseline Hormonal Laboratory Values	**Median (Range)**
AMH (ng/ml)	3.26 (0.44-12.87)
FSH (mIU/mL), cycle day 2	5.65 (1.7-9.5)
E2 (pg/mL), cycle day 2	43 (19-163)
Stimulation Details	**Median (Range) or Percent (Count)**
Initial FSH Gonadotropin Dose (IU)	150 (50-300)
Initial HMG Gonadotropin Dose (IU)	75 (0-150)
Total Gonadotropin Dose (IU)	2375 (825-8075)
Duration of Stimulation (days)	10 (8-21)
Maximum E2 Level (pg/mL)	3220 (943-6969)
Number of Cancelled Cycles	5% (1/22)
Reason for Cancelled Cycle	Low Response
Cycle Protocol	
Antagonist	95% (21/22)
Low-dose leuprolide acetate downregulation	5% (1/22)
Trigger Details	
10,000 U hCG only	9% (2/22)
1000 U hCG + single GnRH-a	9% (2/22)
1000 U hCG + double GnRH-a	59% (13/22)
5000 U hCG + double GnRH-a	14% (3/22)
10,000 U hCG + double GnRH-a	4.5% (1/22)
Post Trigger LH level (mIU/mL)	82.9 (0.9-187.2)
LH < 20mIUm/mL	15.8% (3/19)
LH 20mIUm/mL - 40mIUm/mL	10.5% (2/19)
LH > 40mIUm/mL	73.7% (14/19)
Post Trigger hCG, level (mIU/mL)	55 (2.66-215)
hCG < 40mIU/mL	47% (9/19)
hCG ≥ 40mIU/mL	53% (10/19)
Retrieval Outcomes	**Median (Range) or Percent (Count)**
Total oocytes retrieved, number	22 (5-59)
<10 oocytes retrieved	14% (3/21)
10-19 oocytes retrieved	19% (4/21)
≥ 20 oocytes retrieved	67% (14/21)
Maturity Rate	73% (19%-100%)
Number of MII oocyte cryopreserved	15 (3-35)
<10 MII oocytes	33% (7/21)
10-19 MII oocytes	43% (9/21)
≥ 20 MII oocytes	24% (5/21)
Number of MI oocyte cryopreserved	2 (1-9)
Any MI cryopreserved	71% (15/21)
Number of GV oocyte cryopreserved	3 (1-20)
Any GV cryopreserved	81% (17/21)

AMH, Anti-mullerian hormone.
FSH, Follicle stimulating hormone.
hCG, Human chorionic gonadotropin.
E2, Estradiol level.
HMG, Human menopausal gonadotrophin.
Antagonist, Gonadotropin-releasing hormone antagonist protocol.
Low-dose leuprolide acetate downregulation, Provera for 10 days, leuprolide acetate 10U starting day 6 of provera reducing to 5U on cycle day 2 with initiating of gonadotrophins.
GnRH-a, Gonadotrophin releasing hormone agonist.
LH, Luteinizing hormone.
Total oocytes, Total oocytes (MII, MI, GV).
MII, Metaphase II oocytes.
MI, Metaphase I oocytes.
GV, Germinal Vesicle oocytes.

Most patients cryopreserved at least 10 mature oocytes (67%, 14/21) with many patients additionally freezing immature oocytes. All retrievals resulted in frozen Metaphase II (MII) oocytes (median 15, range 3-35). Two patients (age range 12-14 years) completed second oocyte cryopreservation cycles due to first cycle low maturity rate (23% MII, 3/13) and poor response (5 MII retrieved). Nine additional oocytes (6/9 MII oocytes) were cryopreserved for the first individual and 15 additional oocytes (8/15 MII oocytes) were cryopreserved for the second individual in the second oocyte cryopreservation cycle.

Complications

Four cycles were complicated by mild or moderate ovarian hyperstimulation syndrome (OHSS) (**Table 3A**). All patients were managed with outpatient supportive care. Two of the patients had the highest maximum E2 (6969 pg/mL, 6091 pg/mL) and highest oocytes retrieved (59 oocytes, 43 oocytes). Notably, both patients with a history of testosterone use prior to stimulation experienced OHSS. One of the patients was on the low-dose down-regulation protocol with leuprolide acetate who required a hCG only trigger.

Subgroup Analysis

Patients who received oral contraception, leuprolide, and/or testosterone were sub-divided to evaluate if fertility outcomes differed in those who received pre-consult affirming therapy compared to those who did not. Patients who received oral contraception, leuprolide, and/or testosterone prior to oocyte cryopreservation were significantly older at time of stimulation than those who had not started these gender affirming treatments (p=0.02). There were no other significant differences between groups regarding days of stimulation, AMH, day two FSH or E2, maximum E2, oocyte maturity (MII, MI, GV, or total), number of oocytes retrieved, or total gonadotropin dose. Importantly, all patients with prior testosterone exposure were successful in ovarian stimulation and in cryopreserving mature oocytes. (**Supplemental Chart A**).

DISCUSSION

Oocyte cryopreservation is a safe and viable option for AYA transmen to preserve their fertility and is an important consideration for providing comprehensive care to the growing transgender youth population. This case series demonstrated several important facets to AYA transmale fertility preservation care including: 1) the importance of referral networks; 2) the desire for expedited gender affirming treatments with or without oocyte cryopreservation; and 3) the feasibility and safety of oocyte cryopreservation alongside dysphoria protecting protocols. Our results showed that in-network hospitals referrals were critical in capturing potentially interested AYA transmen who contacted, consulted, and underwent oocyte cryopreservation, many of whom were unsure of their future desire for biologic children and/or had already begun gender affirming treatments including gender affirming surgery, menstrual suppression with pubertal blockers or oral contraceptive pills, and/or testosterone. Expedited initiation of testosterone remained one of the most common goals regardless of age across patients, and often drove decision-making surrounding pursuit of fertility preservation. To our knowledge, this study is the largest published case series describing the experience of AYA transmen through their journey through oocyte cryopreservation and may provide a foundation for solidifying best practices for AYA transmen who are considering fertility preservation with oocyte cryopreservation.

Low prevalence of fertility preservation has been attributed to cost of treatment, concerns about discrimination, discontinuation or delay of gender-affirming hormonal therapy, or worsening gender dysphoria (15–17, 19, 33). Our cases shared similar barriers relating to discontinuation or delay of hormonal therapy, though many of our patients were still able to proceed with expedited gender affirming treatments following oocyte cryopreservation. Desire for family building and fertility have previously been demonstrated as valuable to many transgender individuals, though desire for biologic children may be lower in transgender youth with greater uncertainty as to whether this opinion will change in the future (12, 16, 19, 21, 33–35). Our cohort shared similar views for uncertainty surrounding biologic parenthood, with an overall lower percentage of those certain of their desire for genetic children (12, 16, 21, 29).

Previous studies have also found need for mental preparation and dysphoric triggers during cycling, though no overt episodes were cited during this current study. The process of oocyte cryopreservation can be a highly feminizing experience with the administration of hormones to increase endogenous estrogens, the possibility for feminizing effects of estrogens, the need to discontinue testosterone or other gender affirming hormonal treatments, and the resumption of menses before beginning the process (9, 23). Health care providers can alleviate distress discussing potential dysphoric events prior to cycle initiation, by using gender-neutral language and preferred pronouns and by incorporating supportive individuals such as friends, family members and partners into the process (23). While none of our patients utilized aromatase inhibitors and experienced minimal dysphoric events, letrozole, when taken during a cycle, can maintain low serum estradiol levels, minimize pubertal development, and prevent gender dysphoria symptoms (36, 37). Monitoring with transabdominal ultrasound can additionally temporize the feminizing character of oocyte stimulation in transmen, for which the majority of our patients opted (9, 16, 31). Using a random start approach for ovarian stimulation can enable patients to proceed with their cycles without needing a menstrual bleed, which may in turn reduce gender dysphoric triggers and expedite timing of cycle initiation (15, 38).

The effect of long-term general affirming testosterone on future reproductive capacity is largely unknown, with even less known about fertility in transgender individuals who have had puberty halted with GnRH agonists. In our testosterone cohort, we found no significant differences in fertility outcomes, though the number was very small. In comparison, current data on the impact of oocyte cryopreservation outcome in transmen remains mixed, with one study finding those previously exposed to testosterone having lower total oocytes retrieved and lower maximum E2 whereas another cohort study that transgender men had overnight increased number of oocytes retrieved but required elevated total gonadotrophin doses (26, 30). As this field grows, the understanding of the impact of testosterone will provide further counseling tools and options for fertility in older transgender individuals. There are no prior studies on the impact of pubertal suppression on fertility preservation outcomes.

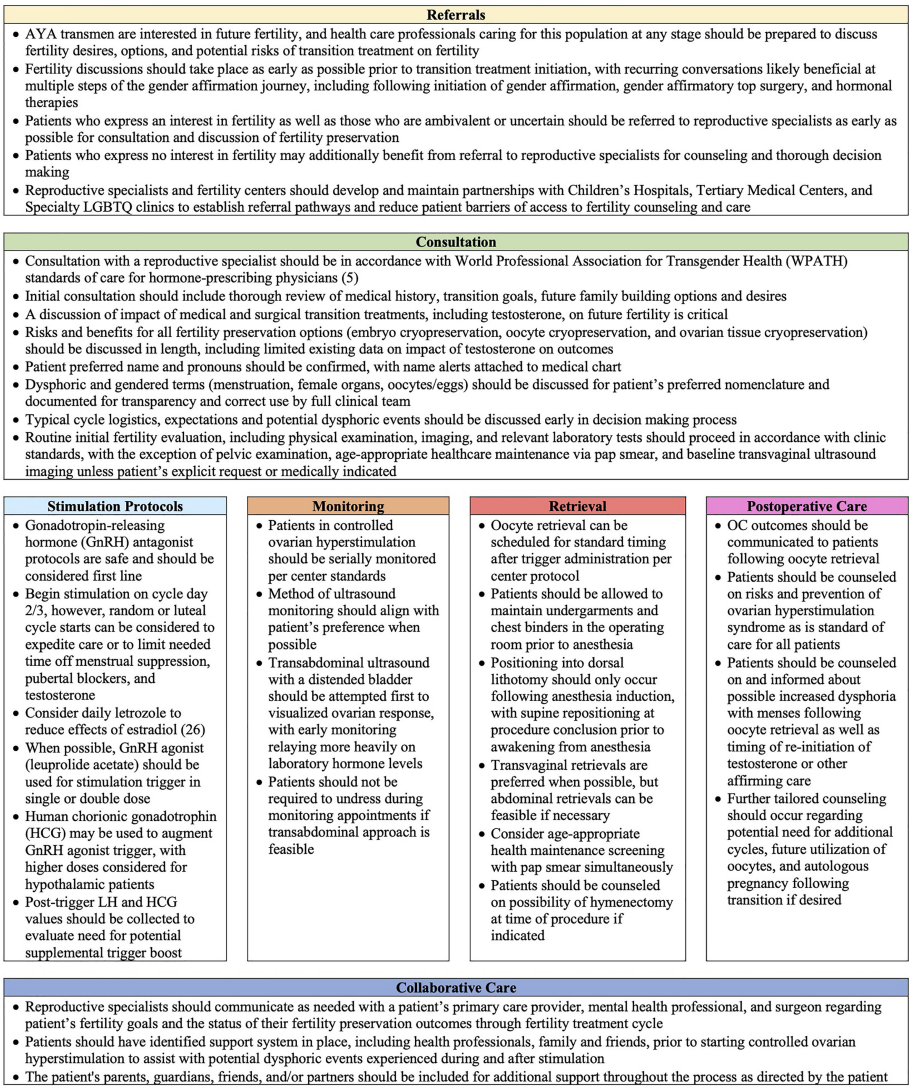

FIGURE 2 | Lessons Learned from Adolescent and Young Adult Transmen Undergoing Oocyte Cryopreservation.

Utilizing the insights evaluated, we highlight key lessons learned for comprehensive care for AYA transmen who may consult or utilize oocyte cryopreservation, as seen in **Figure 2**. These recommendations are modeled from WPATH guidelines, but refined to focus on transparency of the process and specific actionable methodology for reproductive specialists to follow (5). AYA oncologic guidelines for fertility preservation were additionally reviewed for best practices that may be translatable for AYA transmen, including expedited care *via* random and luteal cycle starts and utilization of letrozole during ovarian stimulation to limit E2 to minimize pubertal development and prevent gender dysphoria symptoms (27–29, 37–40). We incorporate into our lessons medical ethics principles of nonmaleficence, beneficence, autonomy as well as the WPATH tenet for creating a safe and supportive environment to maximize the overall health, psychologic well-being, and self-fulfillment of transgender patients (5). Lessons learned are highlighted across seven main pillars for AYA transmen for best practices regarding fertility preservation and oocyte cryopreservation: 1) Referral 2) Consultation 3) Ovarian stimulation Protocols 4) Stimulation Cycle Monitoring 5) Oocyte Retrieval 6) Postoperative Care 7) Collaborative Care. This framework is a starting point that should be adapted and improved upon by a larger, more compressive collaboration of subject matter experts for standard of care guidelines. Our goal in bundling lessons from our institution's experience with fertility preservation and oocyte cryopreservation is to help expand the comfort of providers and thereby access to care for AYA transmen who desire fertility preservation until standard guidelines are expanded. There are many ways to build

families for gender nonconforming individuals, and fertility preservation may not be the right option for all AYA transmen. However, it is crucial to enable gender affirming youth the opportunity to be educated about their fertility options for utilizing their own gametes.

The primary strength of our study is its inclusion criteria of a greater than 10 year time span, allowing for the largest published case series on transgender adolescents undergoing oocyte cryopreservation. This study broadly assesses multiple oocyte cryopreservation cycles to gain insights into standardized practices tailored towards AYA transmen. Our study is limited in its generalizability as it was completed at a single institution. Furthermore, our population was limited in their prior exposure to pubertal blockers and testosterone, making it difficult to draw conclusions on this specific patient population. More comprehensive and expansive research is needed to evaluate the outcomes and experiences of transmen who are on testosterone or pubertal blockers. Additionally, its retrospective design and reliance on chart documentation limited our ability to further explore decision-making surrounding fertility preservation, barriers to consultation or fertility preservation, regret/emotional stress/physical comfort during consultation or oocyte cryopreservation, and satisfaction with the process. In particular, we were not set up to use standard questions and instead relied on chart review of primary care, social work, and infertility specialist documentation surrounding episodes of gender dysphoria, expressed concerns with fertility goals, and reasons for declining fertility consultation or preservation. This methodology is limited in our ability to capture gender dysphoria episodes or reflect the complicated decision-making surrounding the OC process. This study focused on patients who pursued formal REI consultation and may be skewed towards those more likely to undergo oocyte cryopreservation. Further oocyte cryopreservation outcomes related research is needed to provide evidence-based and patient-centered care surrounding AYA fertility preservation. Further work is needed to standardize and tailor oocyte cryopreservation protocols towards the unique needs of AYA transmen, including the development of pubertal pathways as well as pathways for those previously exposed to testosterone or pubertal blockers. Finally, and possibly most importantly, the young nature of oocyte cryopreservation in this population has limited outcome data from oocyte utilization and fertilization and so the true reproductive potential of these cryopreserved gametes is unknown.

In conclusion, we present the largest published case series of oocyte cryopreservation in AYA transmales and identify lessons learned for best practices based on the experiences of our AYA transmale patients, as a tool for AYA patients and healthcare professionals caring for transgender and gender-nonconforming adolescent and young adults. These lessons may help inform more standard guidelines to empower both patients and providers to better understand fertility consultation, fertility preservation, and oocyte cryopreservation, provide transparency surrounding the oocyte cryopreservation process, and ease the decision to pursue an oocyte cryopreservation cycle in parallel to their gender-affirmatory care.

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 New York University Grossman School of Medicine Institutional Review Board (i13-00389). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JB and MF conceived and designed the study. FB completed the statistical analysis and drafted the manuscript. JS gave critical revision for important intellectual content. All authors interpreted the data, revised the paper for important intellectual content, and approved the final version. All authors contributed to the article and approved the submitted version.

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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.2022.873508/full#supplementary-material

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Oncofertility and Fertility Preservation in Cancer Patients Across the Twitterverse

Nayeli A. Martinez-Ibarra[1†‡], *Yuly A. Remolina-Bonilla*[1‡], *Hector H. Buerba-Vieregge*[1‡], *Regina Barragan-Carrillo*[1‡], *Francisco J. Castro-Alonso*[2‡], *Samantha Mateos-Corella*[1‡] *and Maria T. Bourlon*[1*‡]

[1] Department of Hematology and Oncology, Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", Mexico City, Mexico, [2] Department of Internal Medicine, Hospital Regional de Alta Especialidad de Oaxaca, San Bartolo Coyotepec, Mexico

Purpose: Infertility is a major problem affecting children, adolescents, and young adults (AYAs) with cancer, either due to the disease itself or because of oncologic treatment. Oncofertility (OF) focuses on counseling cancer patients about fertility risks and preservation options. However, OF and fertility preservation (FP) conversations on Twitter and their impact are unknown. We aim to characterize the users and type of content of these conversations.

Materials and Methods: This observational study analyzed tweets with the hashtags "#Oncofertility" and "#FertilityPreservation" over eight months. We classified Twitter accounts by user type and country. Tweets were categorized by content type, and retweets and likes were quantified. Descriptive statistics were used for analysis.

Results: A total of 399 tweets from 223 different accounts were evaluated. Twitter accounts comprised 22 countries and stemmed from high, upper-middle, and lower-middle-income countries in 86.5%, 5.4%, and 6.3%, respectively; no accounts from low-income countries were found. Accounts were mostly from physicians (37%) and healthcare centers (20%); we did not find any patient accounts. The most common content category was informative tweets directed to patients (30.8%), followed by discussion/sharing of medical papers (25.6%). Only 14.5% of tweets contained information about children and adolescents. Still, only 4.5% were aimed at children. Retweets were absent in 16.5% of the tweets, and 80.7% did not have comments.

Conclusion: OF and FP discussions on Twitter were limited to interactions among medical professionals. Also, advocacy groups showed limited activity on social media. Even though a significant proportion of tweets directed to patients were found, no active involvement of patients was observed. Finally, limited number of tweets (4.5%) were directed to children and adolescents. There is a need to raise awareness about the effects

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Martin Angel, Alexander Fleming Specialized Medical Institute, Argentina

***Correspondence:**
Maria T. Bourlon
maitebourlon@gmail.com

[†]*First author*

[‡]*These authors have contributed equally to this work*

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of cancer on fertility in this group. Currently, Twitter is not a resource of information for children and AYAs with cancer who need OF counseling and fertility preservation. Our results open a debate on how to promote the use of social media in the future to improve the quality of OF information available, awareness, and care since there is an unmet need for fertility preservation access in young cancer patients.

Keywords: oncofertility, fertility preservation, cancer, infertility, children, AYAs, Twitter ®

1 INTRODUCTION

During the last decade, overall cancer incidence has increased among children, adolescents, and young adults (AYAs), but mortality has declined (1, 2). With successful treatments and increased survival, patients develop long-term treatment related toxicities. Radiation therapy and chemotherapy can destroy ovarian or testicular tissue and disrupt sex hormone production, increasing the risk of infertility (3). Patients have expressed limited knowledge and distress on fertility impact of cancer therapy (4–7). Patients and their families should receive an individualized assessment of gonadotoxic risk as early as possible after cancer diagnosis, and timely interventions should be performed to protect their reproductive goals. Oncofertility (OF) focuses on providing information and discussing the fertility issues, managing related complications, and bringing fertility preservation (FP) options to patients to maintain their reproductive potential (8). In recent times, OF has finally become a firmly established discipline and has been stated as a universal right (9).

Children and AYAs with cancer are the focus of OF counseling, and they report a higher need for information in virtual media (10). Currently, social media is increasingly uptaken by patients to obtain medical information and has become a burgeoning means of interaction between healthcare providers, healthcare centers, patients, and caregivers (11–15). Almost one-third of patients use social media for health-related reasons, including information, advice, and social support (11). In 2021, approximately 70% of American adults reported using any social media platform. YouTube and Facebook are the most employed, but there is an increase in the popularity of Twitter, especially among young adults. AYAs have the highest rates of social media use among any age group. Users aged between 18- and 29-years account for the 39% of Twitter users (16). This platform could empower young patients' by increasing their medical knowledge and encouraging them to discuss their doubts and decisions with doctors. Informed patients have better disease awareness, higher adherence, and thus better clinical outcomes (17–19). Patients' digital resources also increases patients' participation in advocacy groups, helping others with the same condition and enhancing their satisfaction (20).

Cancer specific online communities follow particular interests and thus, interactions between diverse stakeholders including patients, families, healthcare providers, advocates, and policymakers take place. Several studies have examined the content of Twitter conversations regarding cancer, primarily discussions about specific tumors like breast, prostate, and lung cancer, which are the leading cancers among men and women globally (21–26). Cancer information on Twitter includes awareness, prevention/risk information, advice seeking, emotional support, cancer treatments, as well as disease outcomes and expectations (23–26). These interactions provide opportunities for non-clinicians, oncology professionals, cancer patients, and those who assist them to share information, advice, and support.

There are no published reports examining OF users and FP conversations in Twitter, nor users' characteristics pertaining to discussions about OF. The aim of this study was to explore this field and its content on Twitter, determine the demographics of the origin of the tweets, the dissemination and impact generated by the shared information, and assess who is tweeting about it.

2 MATERIALS AND TWEETS

Twitter is an information network made up of short messages known as "tweets" with a 280-character limit with over 206 million daily active users worldwide reported in 2021 and increasing daily (27, 28). Tweets can be liked, forwarded ("retweeted"), and replied. When users want to connect to other tweets containing a specific word or topic, they must look for a hashtag (a keyword or phrase preceded by the # symbol) (27). Cancer information on Twitter includes awareness, prevention/risk information, advice seeking, emotional support, cancer treatments, as well as disease outcomes and expectations (23–26).

In contrast with other malignancies with solidified social media outlets, such as breast (#BCSM), prostate (#PCSM) and lung cancer (#LCSM), Oncofertility has not a fully established presence in social media (15). We limited our search to evaluate the #Oncofertility and #FertilityPreservation hashtags as we considered those were the two that would be more accessible and trackable for the overall population. Indeed, including other hashtags in our search (as #Cancer), would yield a higher sensitivity, but will lower the specificity for the indented research question.

This was an observational study. Twitter' search engine was used to find tweets. Original tweets and cited tweets with the hashtags of interest (#Oncofertility and/or #FertilityPreservation in cancer), in English, with any type of format (text, image, and video), posted within the period between January 1st to August

31st, 2020, were included. Tweets that did not meet the inclusion criteria or had incomplete information were excluded, and duplicated tweets were eliminated.

Accounts from each tweet were classified based on their profile information into the following categories: 1) physicians; 2) other healthcare providers (nurses, psychologists, and fertility counselors); 3) healthcare centers (hospitals, clinics, etc.); 4) professional organizations and societies; 5) medical journals; 6) continuing medical education; 7) patient education; 8) advocacy; 9) patients and 10) miscellaneous (accounts not able to be classified). The medical specialty was also considered. The author's accounts' country of origin was documented and classified based on the 2021 World Bank Data group classification (29). Each tweet's type of content was categorized according to previously published studies (26, 30–32).

All tweets were independently reviewed and classified by two reviewers into one of the following categories: 1) discussion/sharing of papers published in medical journals 2) networking among healthcare professionals; 3) diffusion, sharing, and discussion of meeting presentations and/or invitations to webinars; 4) information for healthcare providers; 5) information for patients; 6) opinions/experiences tweeted by personal accounts; 7) others.

Inter-rater agreement was calculated with Cohen's κ-coefficient. All hashtags contained in each tweet were captured and reviewed to evaluate which ones were the most related to our keywords. Public metrics for each tweet (number of retweets, likes, and comments) were collected to assess their dissemination potential. Descriptive statistics and Chi-square test were used for statistical analysis using SPSS version 25 (SPSS, Chicago, Illinois).

3 RESULTS

3.1 Source of Tweets

A total of 674 tweets were initially captured. After the elimination of duplicates, 399 individual tweets were reviewed. All tweets were written in English and came from 223 accounts. We were able to classify accounts' country of origin in 98.2% of cases. Accounts from 22 countries were documented. According to the World Bank group, classification came from high, upper-middle, and lower-middle-income countries, in 86.5%, 5.4%, and 6.3% respectively. No accounts from low-income countries were found **(Figure 1)**. Similar to the accounts' country of origin, most tweets originated from high-income countries. A complete list of each country's contribution can be found in **Supplement Table 1**.

The highest percentage of accounts belonged to physicians (37%), followed by healthcare centers (20%), other healthcare providers and professional organizations and societies (10% each one), patients education accounts (6%), continuing medical education accounts (4%), medical journals (4%) and advocacy accounts (3%); miscellaneous accounts comprised 6% of cases and no accounts from patients were found. Among the physician accounts' subgroup, the most common identifiable medical specialties were Obstetrics & Gynecology (28%), followed by Medical Oncology (23%) and Urology (17%) **(Figure 2)**.

Gender could be determined in more than half of the accounts (51.5%), of those, 60.8% were women and 39.2% were men. Women tended to tweet more about personal opinions and experiences (70.5%), sharing/discussion of medical papers (35.3%) and information for healthcare providers (32.2%) than men (p= 0.036).

3.2 Tweets Content Analysis

Type of content was classified for all 399 tweets, with an inter-rater agreement of 83% (κ = 0.83; P = <0.001). Concordance was higher for tweets including discussion/sharing of papers published in medical journals (92.3%) and for the dissemination of information for patients (90.3%), and lowest for networking among healthcare providers (77.8%). **Table 1** shows the proportion of each tweet type of content and a representative example. Most tweets were about the dissemination of information for patients (30.8%), followed by

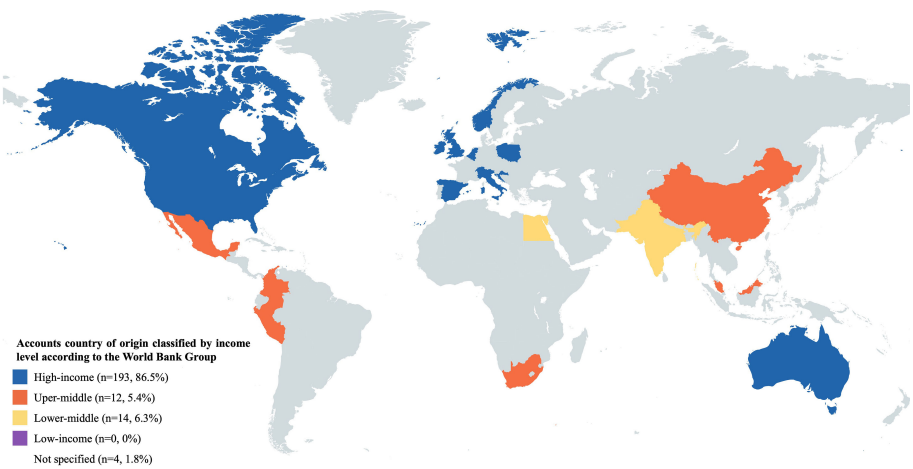

FIGURE 1 | Country of origin of Twitter accounts of participants in OF and FP tweets classified by income level according to the World Bank Group.

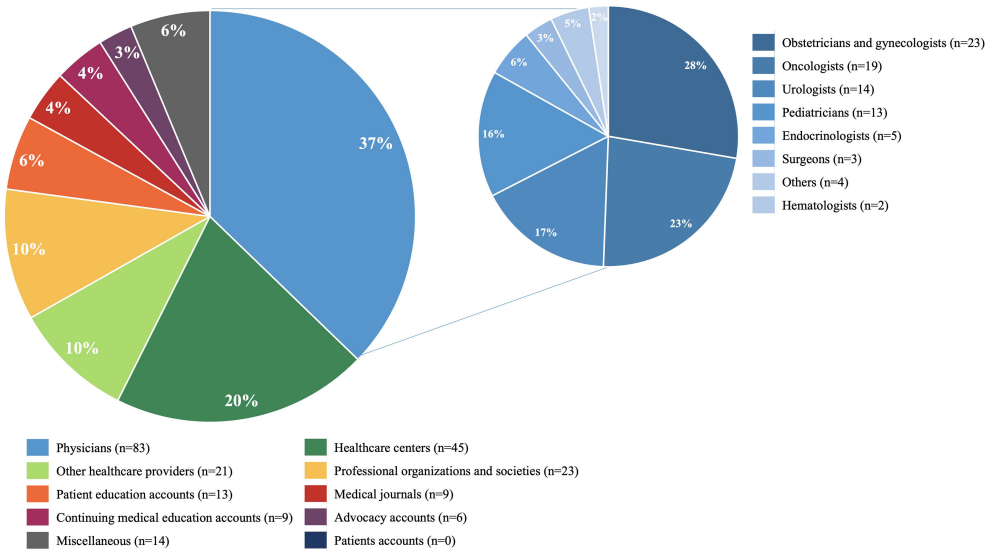

FIGURE 2 | Distribution Twitter accounts according to the type of user holder (larger pie) and medical specialty distribution if the user was a physician (smaller pie).

the discussion/sharing of papers published in medical journals (25.6%), information for healthcare providers (14.8%), opinions/experiences tweeted by personal accounts (11.0%), sharing and discussion of meeting presentations and/or invitations to webinars (7.5%) and lastly, networking among healthcare providers (5.5%). There was insufficient information to classify 4.8% of tweets. About the tweets that were directed to patients (n=123, 30.8%), most of them contained general information (n=82). There were 22 tweets intended at women, one directed at men, and 18 directed at children and adolescents (**Table 2**).

Links to websites were included in 66.6% of the tweets and 69.4% contained at least one image or video. Statistical differences regarding the type of content and tweet authors were found ($p<0.001$) as well as in medical specialties ($p=0.006$). In the category of discussion of papers, 50% of tweets were posted by physicians and 17.6% by medical journal accounts. Networking tweets were commonly created by professional organizations and societies (68.2%), information for patients tweets by institutes or medical centers (41.5%), and diffusion of meeting or webinars, information for

TABLE 1 | Classification of OF and FP related tweets according to their content and a representative tweet of each category.

Type of content	n=	%	Representative tweet
Information for patients	123	30.8	Some cancer treatments may affect #fertility, but there are preservation options. Before treatment begins, ask how it may affect your fertility, and discuss concerns with your health care team. https://fal.cn/385og#oncofertility #infertility #womenshealthweek
Discussion of medical papers	102	25.6	Great series of articles in @XXXXX on #fertility preservation in #cancer patients: #oncofertility is a universal right and a #GlobalOncology priority. Congrats to all the authors, very well done! @XXXXX @XXXXX #OncoAlert @XXXXX https://ascopubs.org/doi/full/10.1200/GO.19.00337#.Xl_yE6437Eg.twitter
Information for healthcare providers	59	14.8	Comparing Options for Ovarian Tissue Cryopreservation to Preserve Fertility in Pediatric Patients With Cancer https://ascopost.com/news/january-2020/ovarian-tissue-cryopreservation-to-preserve-fertility-in-pediatric-patients-with-cancer/#pedonc #oncology #cancer #oncofertility
Personal opinions/ experiences	44	11.0	RT "Preserving fertility in cancer during a crisis may sound "elective", but to the young adult with cancer, it can mean hope in the face of a future clouded by uncertainties. Let's safely care for this vulnerable population while upholding our social obligation. @XXXXXX" I wholeheartedly agree! #oncofertility #ChildhoodCancer #AYAcancer
Meetings' diffusion	30	7.5	The next #fertilityfocus takeover will be on the 16th of July with the brilliant @XXXXX, Join XXXXX as he takes over the Urology News handle to discuss oncofertility. If it's like his last takeover, it's going to be good. #Fertilitythursday
Networking among healthcare professionals	22	5.5	Are you a service provider working with people with cancer? Can you spare 10 minutes to take our survey? #oncology #alliedhealth #ruralhealth #psyonc #supponc #oncologynurses #oncofertility #lgbtonc #oncorn #radonc #surgonc #radonc #onconav
Others	19	4.8	POWER THROUGH: cancer at age 3; e-learning at age 10…what kind of mom do you think she will be? #ChildrenSurvivingCancer #Oncofertility

"XXXXX" was used to censor users' account to protect their privacy.

TABLE 2 | Tweets with information directed to patients and examples of tweets directed to, women, men and children and adolescents.

	n=	%	Representative tweet
General information	82	66.7	Some cancer treatments may affect #fertility. Before treatment begins, ask how it may affect your fertility and discuss concerns with your health care team. http://bit.ly/FertilityConcern #oncofertility #infertility #womenshealth #IWD2020
Women	22	17.9	With the field of medicine advancing every day, #oncofertility joins the two fields of oncology and #gynecology to provide cancer survivors with the chance of increasing their reproductive level. To know more visit- https://buff.ly/2NUnbej
Men	1	0.8	If you have a cancer diagnosis, did you know you may be able to freeze your sperm before treatment? Let's start increasing awareness of the #fertilitypreservation option #WorldCancerDay
Children and adolescents	18	14.6	Did you know treatment for pediatric cancers is a common cause of infertility? Help me promote #oncofertility awareness and support for this amazing event. Only a few tickets left #sharethelove @XXXXXXX https://instagram.com/p/B70qb_8lP4I/?igshid=3iqov27e33ee

healthcare providers and personal opinions tweets mainly by physicians in 30.0%, 35.6% and 65.9%, respectively.

3.3 Reach and Dissemination

The median number of all tweets per user was 9.8 (range 11 – 225,700), and the median number of OF and fertility preservation-related tweets per user was 2.

The median users' followers were 13,594 (range 9 – 1,267,484), whilst the median of followed accounts was 1,041 (range 0 – 14,600). We found 19 accounts with less than 100 followers (8.5% of users) and 24 accounts with less than 100 followed accounts (10.7%). Only 2 accounts had 0 followers (0.8%). Therefore, the probability of fake accounts is considered low (≤1%).

Among OF tweets, the median number of retweets was 2 (range 0 – 95), with a total of 802 retweets, whereas the median number of likes was 6 (range 0 – 66), with a total of 2,488 likes.

A great percentage of the tweets (47.3%, n=189) did not receive any retweet; 16.5% (n=66) received one retweet, 10.5% (n=42) received two retweets and 15.0% (n=60) received three or more retweets. We found that 23% (n=92) of tweets did not receive any likes, 25.0% (n=100) received at least one or two likes and 41.8% (n=167) received three or more likes. Concerning comments or replies, most of the tweets (80.7%) did not get any, 11.5% received one reply, and 7.8% received at least two or more replies.

Among 802 retweets, the majority (n=246) belonged to the discussion/sharing of papers published in the medical journals category; the least retweeted category was personal opinions/experiences (n=58). As to likes, at the top of all the categories, we found the discussion/sharing of papers (n=793), followed by dissemination of information to patients (n=423), and the least liked category was networking among healthcare professionals (n=175) **(Figure 3).** The most frequently liked and retweeted tweets were posted by physicians (34.6% and 34.6%, respectively), followed by professional organizations and societies (20.1% and 20.1% respectively). We found no statistical differences in content, authors, and dissemination surrogates (retweets, likes, commentaries) in the participants' countries according to World Bank income level since most of them were high-income countries.

The hashtags more associated with our keywords were #Cancer, #Fertility, #Infertility, and #FertilityMatters. The hashtag with the COVID-19 word was included in 5.2% (n=21) of tweets.

3.4 OF and FP Information Shared on Twitter Regarding Children and Adolescents

A total of 58 tweets (14.5%) were assessed from 47 distinct accounts from 8 different countries. The most common author category (n=19, 40.4%) was physicians and the predominant country of origin of the accounts was the United States (n=19, 72.3%). The most popular type of information shared was discussion/sharing of papers published in medical journals (n=18, 31%) and dissemination of information for patients (n=18, 31%), tweets belonged to this category were categorized

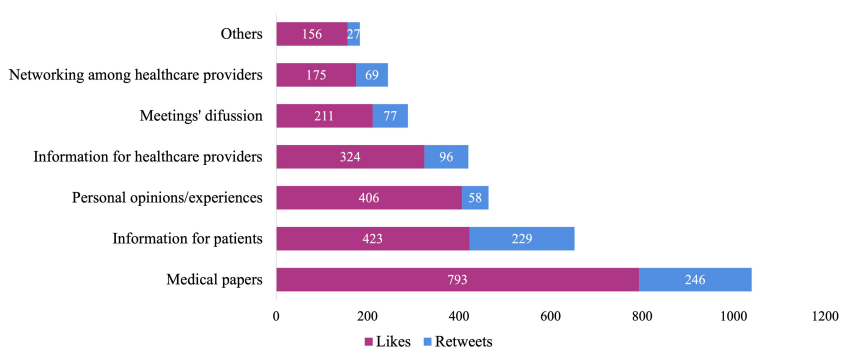

FIGURE 3 | Reach and dissemination of tweets organized by content.

into awareness (n=7), FP programs (n=7) and advice and support (n=4) **(Figure 4)**.

These tweets received 272 likes and 70 retweets, with 49 of them (84.5%) receiving no response. The discussion/sharing of papers published in medical journals received 99 likes and 38 retweets, while the dissemination of information for patients received 81 likes and 23 retweets.

4 DISCUSSION

To our knowledge, this is the first study exploring OF and FP tweets, and impact generated. Most tweets belonged to accounts from high-income countries, which aligns with the adoption, widespread, and dissemination of OF in these countries. Social media use is beyond national wealth and internet availability and is significantly linked to the population's age: a youthful population (i.e., the AYA-age group) contributes to greater social media use in developing countries *vis-à-vis* countries with higher incomes but with a low rate of social media adoption and a more aged population (33).

Low-income countries' lack of engagement may be explained by more than just their income level. The disparities in the income level participants' countries reflect known barriers in the equitable access to the OF field, barriers beyond internet access. Such obstacles include a lack of referral pathways, cost-based access limitation, limited health literacy, lack of training or awareness among medical professionals, cultural or religious constraints, and a lack of consensus about the best way to deliver information to patients (34, 35). Recent clinical practice guidelines published by the PanCareLIFE Consortium state interventions to overcome the barriers, among which written, and online educational resources have shown to increase discussion rate of infertility risks and options to preserve fertility to AYA cancer patients (36). We observed an important participation of India in our study **(Supplement Table 1),** it was the fourth country to tweet about OF and FP. In the field of OF, India has shown a significant interest in overcoming several barriers to improve scientific knowledge, service delivery, advocacy, and research efforts (37).

Almost 25% of patients aged 10 to 12 years old and nearly 40% of patients aged 16 to 18 years old expressed a greater need for cancer education (38). We need to increase cancer knowledge and reduce the fear and stress caused by these patients' future and we believe that Twitter with its large proportion of young users is a powerful social media tool for starting conversations about fertility concerns; thus, the OF community should make direct efforts to leverage discussions in this setting. Social media can help to bridge socioeconomic disparities.

OF conversations were dominated by physicians and mainly were about medical papers. The use of social media by doctors and healthcare providers is well established: surveys report that 72% of oncology physicians or trainees use these platforms for professional development and networking (39). In our study, oncologists were the second most common specialty among physicians' accounts. The popularity of Twitter among oncologists is constantly growing. According to a survey of Canadian oncology physicians and trainees, 72% of respondents used social media (40). Oncologists' involvement in this social media platform has been established in studies published in the Journal of Oncology Practice, with oncologists updating, educating, and expanding knowledge transmission of credible evidence-based information (39, 41). Oncologists on Twitter also promote active patient involvement in cancer discussions (42). Oncologists are expected to participate in OF tweets because they are the ones who prescribe cancer treatments and oversee the implications of treatment side effects during the follow-up and are more conscious about these issues compared with other medical specialists.

The dissemination of information directed to patients was the most popular category, nevertheless we found no active participation of patients in these conversations, nor tweets authored by patients in the study period. The lack of involvement of patients in our study shows a significant

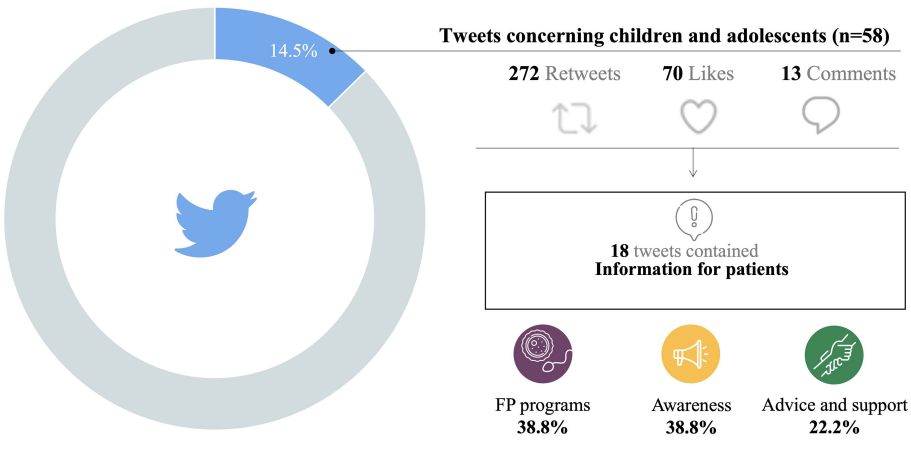

FIGURE 4 | OF and FP information shared on Twitter regarding children and adolescents.

contrast with previously published analyses about Twitter use in other oncology fields. For example, Twitter discussions dealing with breast, lung, prostate, and kidney cancer show a highly engaged community in which most tweets are authored by patients, cancer survivors, and family members. In addition, content is mainly related to cancer diagnosis, treatments, and their side effects and tweets looking for guidance and support (12, 22–26).

The lack of engagement regarding OF social media with the AYA was not expected, as prior reports mentioned that most social media users are aged between 18 and 29 years. Nonetheless a lack of uptake and acceptability of health promotion on social media has been low among young people, with an average of engaged AYA participants ranging from 5 to 15% (43). We hypothesize that AYA's social media use is more of an outlet from their disease than a tool for health-related information access or sharing. Social media use for health-related reasons in AYAs remains a controversial topic. Even though a great amount AYAs use social media, only a small percentage (3.5%) use it for seeking health information. The health-information they tend to look for in social media is about fitness and sexual health (44). Most of the time they spend on their cellphone is because they are passing time or connecting with friends and family (44, 45).

Current social media platforms availability must be also considered. For its nature dependent on words rather than interactive media, Twitter is less attractive to young patients and the general population as a source for learning (23). Young adults found interactive media (for example, video format) easier to learn from. They stated that they could identify accurate YouTube health content and felt that the presenter was honest and relatable. The former contrasts with Twitter, which was rarely used as a source for health information (46).

In addition, only 4.5% (n=18) of all tweets were directed to this population bringing information about FP programs, awareness, and advice and support stressing the need for information created to inform patients, rather than exclusively scientific content aimed at physicians.

As for children with cancer, direct social media interaction is highly unlikely though Twitter, so efforts are aimed at their parents about informing potential infertility risk and OF assessment. Even though we found some tweets aimed at informing parents of children with cancer about infertility risk a similar lack of interaction was observed, like AYAs. In this population, Facebook was the most widely used social media platform by a wide margin; 78% of parents reported using it every day, but only 2% using Twitter daily (47).

We found little engagement of advocacy accounts (3%). OF advocacy groups should take advantage of this platform to improve their reach among potential patients and family members.

Greater participation of females compared to male users was observed. This phenomenon has several potential explanations. The estimated number of new cancer cases in AYA women almost doubled the cases in AYA men in 2020 (8.7% and 4.4%, respectively), thus having numerically more fertility concerns (21). Moreover, FP strategies for females are more technically demanding and time-intensive than those required for male patients, with higher complication rates and lower chance of success which varies between 40 to 61.9%. FP in females also depends on age at retrieval, number of oocytes, and technique (48–51). The complications rates of oocyte retrieval are less than 0.5%, however, these complications can be severe, and life-threatening and should not be underestimated (52). These difficulties might drive female treating physicians to be more likely to discuss fertility issues with their patients, as some studies have shown (53, 54).

Our study has several limitations. There are no standardized methods to perform an analysis of social media; in specific tumor types like breast and prostate cancer, social media traffic is substantially different among different time frames (55). Prior medical research using Twitter have utilized observation timeframes from 22 days to 12 months (32, 56). We considered that the collected information during our timeframe provides a realistic scope of current social media use. As this is the first study of its nature, we considered that these results are vital for the OF community and key for designing interventions to improve social media engagement in AYAs.

Furthermore, Twitter's search engine may have some limitations in the access of all the tweets searched with the hashtag of interest, like a misspelling. Using English keywords might ignore information in other languages, so equivalent words in other common tongues should be performed. Other social media platforms could be used as a dominant way of information, but the way they are designed to make them less trackable than Twitter. Finally, OF and FP conversations may start on Twitter and then migrate to verbal in-office discussions, which cannot be followed by this study.

Share more appealing information for patients and caregivers, such as infographics and videos, promote their active participation in Twitter using surveys to find out what their interests are, and create hashtags and communities to facilitate patient access to specific information are some strategies that could help us in increase knowledge, education, and engagement of patients and caregivers.

Future directions are needed to explore and understand how social media can be used and exploited by physicians, other healthcare providers, advocacy groups, patients, and their families for exchanging information. There is ample opportunity among the OF community (especially to patients and advocates) to use Twitter as a means to improve their reach and leverage fertility discussions.

5 CONCLUSION

Oncofertility and fertility preservation discussions in Twitter were limited to interactions among medical professionals and medical centers, while limited participation of advocacy groups and no active involvement among patients were observed. From a Global Oncology perspective, most tweets came from high-income countries, with limited participation of middle-income

countries and a total lack of participation in low-income countries.

The Oncofertility community needs to implement initiatives directed to create more appealing social media content that captures patients' attention, facilitates their engagement in decision-making, and improves their long term well-being in survivorship. There is a need to raise awareness about the fertility impact of cancer in children and AYAs. These results open the debate whether social media could be used in the future to improve the quality of oncofertility care and suggest that exists a need to identify strategies to increase fertility education for cancer patients and their families.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All procedures performed in this study were in accordance with the ethical standards of the institution. There were no human subjects as part of this manuscript and, therefore no need for informed consent.

AUTHOR CONTRIBUTIONS

Conception and design: MB; Data collection: NM-I, SM-C, and HB-V; Analysis and interpretation of data: NM-I, YR-B, HB-V, RB-C, FC-A, SM-C, and MB; Manuscript writing and approval of final article: All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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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.2022.926668/full#supplementary-material

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EDITED BY
Asma Chattha,
Mayo Clinic, United States

REVIEWED BY
Francisco Prat,
Spanish National Research Council (CSIC), Spain
Mohammad H. Abukhalil,
Al-Hussein Bin Talal University, Jordan

*CORRESPONDENCE
Shou-Long Deng
popo84350746@163.com
Hua Jia
huajia1981@yahoo.com
Wen-Zhi Ma
mawenzhi126@126.com

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Melatonin prevents cyclophosphamide-induced primordial follicle loss by inhibiting ovarian granulosa cell apoptosis and maintaining AMH expression

Juan Feng[1], Wen-Wen Ma[1], Hui-Xia Li[1], Xiu-Ying Pei[1], Shou-Long Deng[2]*, Hua Jia[1]* and Wen-Zhi Ma[1]*

[1]Key Laboratory of Fertility Preservation and Maintenance of Ministry of Education, and Key Laboratory of Reproduction and Genetics of Ningxia Hui Autonomous Region, School of Basic Medical Science, Ningxia Medical University, Yinchuan, China, [2]NHC Key Laboratory of Human Disease Comparative Medicine, Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Comparative Medicine Center, Peking Union Medical College, Beijing, China

Cyclophosphaty -45mide (Cyc) chemotherapy in young female cancer patients is associated with an increased risk of premature ovarian insufficiency (POI). This study was designed to investigate the protective role of melatonin (Mel) as an adjuvant against Cyc-induced POI. Female mice received a single intraperitoneal (i.p.) dose of Cyc (75 mg/kg). Mel protection was achieved in mice after i.p. injection of melatonin (50 mg/kg) every 24 h for four consecutive days prior to chemotherapy initiation and for 14 additional days. Ovarian reserve testing, hormonal assays for follicle-stimulating hormone, luteinizing hormone, and anti-Müllerian hormone (AMH), assessment of the oxidative stress status, and measurement of the relative expression of genes in PTEN/AKT/FOXO3a and mitochondrial apoptosis pathways were performed. The results showed that treatment with 50 mg/kg Mel significantly prevented Cyc-induced over-activation of primordial follicles by maintaining the plasma level of AMH and subsequently preventing litter size reduction in mice treated with Cyc chemotherapy. Importantly, Mel treatment significantly prevented ovarian granulosa cell loss by inhibiting the mitochondrial apoptotic pathway. Identifying the protective actions of Mel against Cyc-induced primordial follicle loss has important implications for fertility maintenance in young cancer patients undergoing chemotherapy.

KEYWORDS

melatonin, cyclophosphamide, primordial follicle, anti-Mullerian hormone, granulosa cell, apoptosis

Introduction

With the increasing number of cancer survivors among children and adolescents, the issue of fertility preservation has assumed greater importance. All young patients with cancer or leukemia should have their fertility prognosis discussed before treatment initiation. The commonly used method of fertility preservation in female children is the freezing of ovarian tissue or unfertilized oocytes. These fertility preservation methods among children and adolescents are in the experimental stage, and none provide 100% effectiveness (1–3). For young female cancer patients who require immediate chemotherapy, finding an adjuvant to protect their ovaries during chemotherapy is one of the best options available to preserve fertility.

There are two main pathways for premature ovarian failure (POF) caused by the depletion of primordial follicle reserves after chemotherapy (4, 5); one is the direct toxic effect of the agent on follicular oocytes and granulosa cells, and the other is indirect over-activation of primordial follicles through damage to growing follicles (6, 7). The second pathway is the result of loss of negative feedback regulation, which inhibits primordial follicle activation (8, 9). Cyclophosphamide (Cyc), a commonly used chemotherapy, has a wide range of effects as a broad-spectrum antineoplastic drug (9, 10). Cyc induces apoptosis of granulosa cells in growing follicles by activating Bax and the mitochondrial apoptosis pathway (11). Granulosa cells of growing follicles proliferate rapidly and are highly sensitive to chemotherapeutic drugs (12).

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β superfamily, which is mainly produced by granulosa cells in secondary and early antral follicles (6, 12–14). AMH is an important negative regulator of primordial follicle recruitment; it inhibits the activation of primordial follicles and reduces the sensitivity of antral follicles to follicle-stimulating hormone (FSH) during the recruitment cycle, thus maintaining a certain number of primordial follicles and growing follicles in the ovary (15). Cyc-induced apoptosis of granulosa cells in growing follicles decreases the level of AMH, reduces the inhibitory effect of AMH on primordial follicle recruitment, and enhances the sensitivity of antral follicles to FSH during cycle recruitment, resulting in a further decrease in AMH levels and indirect over-activation of primordial follicle reserves (12, 16).

Melatonin (Mel) attenuates Cyc-induced loss of primordial follicle loss and is produced by the pineal gland, ovaries, and placenta (17–20). Previously, Mel was found to reduce POF in mice caused by oxidative stress through the sirt1 signaling pathway (21). Mel also has anticancer effects due to its activation of the p53 and p21 signaling pathways, thereby inhibiting cancer cell growth and downregulating the vascular endothelial growth factor receptor to reduce angiogenesis (22, 23). Mel is an ideal adjuvant for chemotherapy because it neutralizes superoxide anions, hydrogen peroxide, and other oxygen-based free radicals.

Studies have shown that Mel protects against damage to the primordial follicular pool caused by cisplatin chemotherapy by inhibiting the PTEN/AKT/FOXO3a signaling pathway (6). However, the mechanism by which Mel prevents ovarian POF after Cyc chemotherapy remains unclear. Here, we investigated the mechanism by which Mel prevents Cyc-induced ovarian POF. We verified that Mel administration successfully rescued Cyc-induced primordial follicle loss by inhibiting ovarian granulosa cell apoptosis and maintaining AMH expression.

Materials and methods

Animals experiments

Six-week-old and three-week-old female Institute of Cancer Research (ICR) mice were purchased from the Experimental Animal Center of the Ningxia Medical University. Animals were fed *ad libitum* and had free access to food and water. They were housed under conditions of constant temperature (21 ± 2°C) and a 12-hr light/dark cycle. All mice were acclimated for three days before the experiment. The experiment was approved by the Animal Care and Use Committee of Ningxia Medical University. Six-week-old and three-week-old female mice were randomly divided into four experimental groups: saline (Sal, control), Cyc, Mel, and Mel + Cyc. There were 25 six-week-old and 48 three-week-old female mice in each group. Mel protection was achieved in mice after intraperitoneal (i.p.) injection of Mel (50 mg/kg body weight; Sigma, St. Louis, MO, USA) or Sal, administered every 24 h for four consecutive days prior to chemotherapy initiation and for 14 additional days to maintain high levels during chemotherapeutic treatment. After the initial 4-day Mel treatment period, mice were injected i.p. with Cyc (75 mg/kg, Sigma) or Sal. The standard dosage of Cyc was selected based on previous studies showing ovarian damage (16, 24). Eighteen six-week-old female mice in each group were euthanized and blood samples and ovaries were collected 14 days after Cyc injection. Seven six-week-old female mice in each group were used for the mating experiment 115 days after the Cyc injection. For three-week-old female mice, ovaries were collected for the evaluation of apoptosis 12 h after Cyc injection (Figure 1A). Thereafter, maintenance of the primordial follicle pool was evaluated by fertility assessments from 115 to 170 days after Cyc injection (Figure 1B). To avoid a false positive of granulosa cell apoptosis during physiological follicular atresia in adult mice, untreated prepubertal three-week-old female mice aged were used, and their follicular

A

Group	Pre-treatment I.P.(day1-18)	Treatment I.P.(day4)	Mouse age	Sample collection time
Sal	Saline	Saline	6W	Day 18
			3W	Day 4.5
Cyc	Saline	Cyclophosphamide 75mg/kg	6W	Day 18
			3W	Day 4.5
Mel	Melatonin 50mg/kg	Saline	6W	Day 18
			3W	Day 4.5
Mel+Cyc	Melatonin 50mg/kg	Cyclophosphamide 75mg/kg	6W	Day 18
			3W	Day 4.5

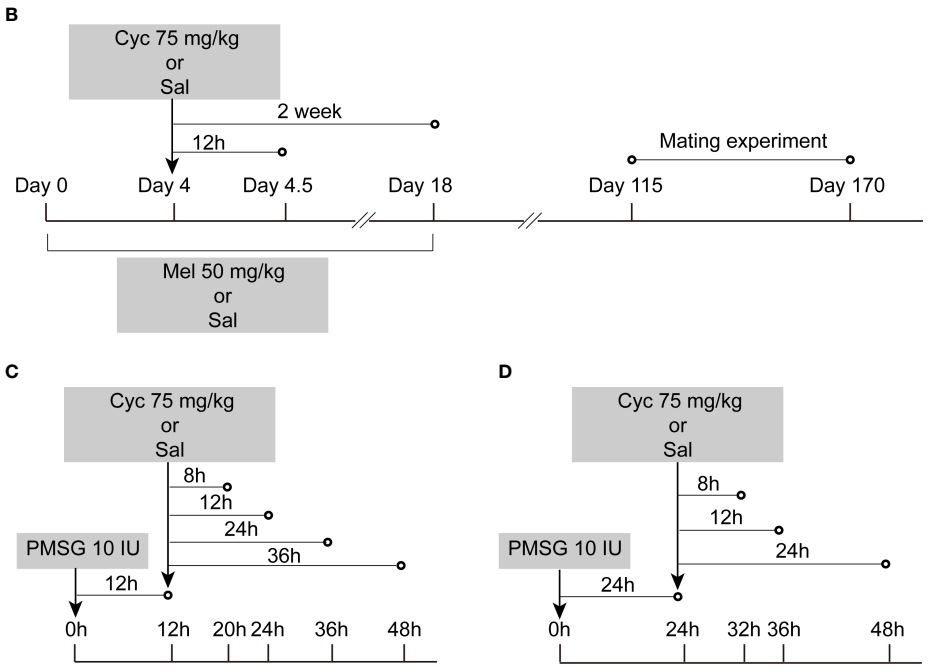

FIGURE 1
Graphic illustration of experimental schedule. **(A)** Mice were randomly divided into four groups. **(B)** Six or three-week-old ICR mice were pre-treated with i.p. injections of Mel or Sal once daily (at 24 h intervals) for four consecutive days before achieving Cyc injection, and for 14 additional days during chemotherapy treatment. On Day 4, mice were injected with Sal or Cyc (75 mg/kg). Blood and/or ovaries were collected 12 h or two weeks after Cyc injection. Fertility assessment was performed 115 days after Cyc injection. **(C)** Three-week-old prepubertal female mice were injected i.p. with 75 mg/kg body weight Cyc 12 h after injection of PMSG. Mice were sacrificed 8, 12, 24, or 36 h after chemotherapy treatment and their ovaries were excised (n = 4). **(D)** Prepubertal female mice were injected i.p. with 75 mg/kg body weight Cyc 24 h after injection of PMSG. Mice were sacrificed 12, 24, or 36 h after chemotherapy treatment and their ovaries were excised.

development was activated by injection of 10 IU of pregnant mare's serum gonadotropin (PMSG). Mice were injected i.p. with 75 mg/kg body weight Cyc 12 or 24 h after the injection of PMSG. They were sacrificed at 8, 12, 24, and 36 h after chemotherapy (the longest time was no more than 48 h after PMSG injection), and their ovaries were excised for apoptosis detection (Figures 1C, D). To study the function and mechanism of Mel intervention on apoptosis of granulosa cells in prepubertal three-week-old mice, 12 h was selected as the interval after Cyc injection in the subsequent experiments (Figure 1B).

Histology and follicle counts

Follicle counting was performed according to previously published methods (25, 26). Briefly, ovaries were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and cut into 5-μm serial sections. The total number of follicles in each ovary was estimated by counting the number of follicles in every fifth HE-stained section and applying a five-fold correction factor. Follicles were divided into five stages: primordial, primary, secondary, antral, and atretic follicles. Only the follicles with apparent oocyte nuclei were counted. Oocytes surrounded by

squamous granulosa cells were classified as primordial follicles. The primary follicles were oocytes surrounded by a layer of cubic granulosa cells. Secondary follicles had two or more layers of cubic granular cells but lacked a lumen. Antral follicles have several layers of granular cells and lumens (27). Atretic follicles had aberrant oocytes and multiple layers of pycnotic granulosa cells.

Immunohistochemistry

Paraffin-embedded sections of mouse ovaries were dewaxed, hydrated, and sealed with 5% normal goat serum after antigen repair. The sections were incubated overnight in anti-Nobox (1:500, Bioss, Woburn, MA, USA; Nobox is an oocyte-specific homeobox gene that plays a critical role in early folliculogenesis (28) and anti-AMH (1:2000, Abcam, Cambridge, UK) at 4°C, and then biotinylated rabbit anti-mouse antibody was added. Immunoreactivity was detected by indirect immunoperoxidase staining (Vector Labs, Newark, CA, USA) with DAB. Images were obtained under a microscope.

Fertility assessment

At 115 days after Cyc treatment, sexually mature ICR female mice were paired with healthy male mice in a 2:1 ratio (26, 29). All male mice used for mating were approximately 12 weeks old and confirmed to be fertile. Female mice were kept with male mice until vaginal plugs were observed in the morning (n = 7). By the fifteenth day after mating, mated female mice that were judged to be pregnant were isolated. After delivery, postnatal mice and mice that failed to conceive were reintroduced to the male for the next round of mating.

Measurement of serum AMH, FSH, and luteinizing hormone

The six-week-old female mice were anesthetized, and blood was collected from the orbital vein behind the eyeball 14 days after chemotherapy. Sera were then separated by centrifugation (4000 × g, 15 min, 4°C) and frozen at -80°C. Serum AMH concentration was measured using a mouse AMH ELISA kit (Elabscience Biotechnology, Bethesda, MD, USA). The serum concentration of FSH was measured using a mouse follicle-stimulating hormone ELISA kit (Jianglai Biotechnology, Shanghai, China). The serum luteinizing hormone (LH) concentration was measured using a mouse luteinizing hormone ELISA kit (Jianglai Biotechnology, Shanghai, China). All measurements were performed in accordance with the manufacturer's instructions.

Measurement of malondialdehyde levels and activities of superoxide dismutase and catalase

Fresh tissue from six-week-old female mice were washed with ice-cold phosphate buffered saline (PBS) solution and weighed. After the weights were recorded, homogenization was immediately performed using a tissue homogenizer on ice and centrifuged. The supernatants were used for the measurements. Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) assays were performed using a spectrophotometer (Jianglai Biotechnology, Shanghai, China). The analysis was performed in accordance with the manufacturer's instructions.

Evaluation of apoptosis

After deparaffinization of three-week-old female mouse ovary slides, the TUNEL BrightGreen Apoptosis Detection Kit Vazyme Code (Vazyme A112-02) was used to perform TUNEL analysis according to the manufacturer's instructions. During apoptosis, intracellular endonucleases are activated, chromatin DNA is specifically cleaved between nucleosomes, and DNA is degraded into 180–200 bp or integer multiple fragments. Nucleotidyl Transferase binds FITC-12-dUTP to the 3' -hydroxyl (3' -OH) end of the DNA molecule break, which can bind FITC-12-dUTP under the action of terminal deoxynucleotidyl transferase. The FITC-12-dUTP-labeled broken DNA can be directly observed with a fluorescence microscope (green represents apoptosis) to reflect apoptosis levels.

Western blot analysis

The ovarian tissues of mice in each group were extracted with RIPA lysis buffer, and the protein concentration was determined using the bicinchoninic acid assay. The total protein from each group was added to the sample buffer for PAGE. Total proteins were separated by electrophoresis on 10–12.5% SDS-PAGE gels. Cytoplasmic and mitochondrial proteins were prepared using a cytoplasmic and mitochondrial protein extraction kit purchased from Sangon Biotech (Shanghai, China) (30). Cytoplasmic and mitochondrial protein extraction was performed according to the manufacturer's instructions. Briefly, the cytoplasm and mitochondria were placed directly in the sample buffer and boiled for 10 min. Protein samples were

separated by electrophoresis on 15% SDS-PAGE gels. After electrophoresis, steps such as closure, membrane transfer, hybridization, and exposure, were performed. The primary antibodies included anti-p-PTEN antibody (1:1000, Affinity Biosciences, Cincinnati, OH, USA), anti-PTEN antibody (1:1000, Affinity, Inc.), anti-p-FOXO3a antibody (1:5000, Abcam), anti-FOXO3a antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-active-Caspase3 (1:5000, Abcam), anti-Caspase-3 antibody (1:2000, Abcam), anti-Bax antibody (1:1000, Cell Signaling Technology), anti-Bcl-2 antibody (1:1000, Cell Signaling Technology), anti-cytochrome C antibody (1:5000, Abcam), anti-VDAC1 antibody (1:5000, Abcam), anti-β-actin antibody (1:5000, Proteintech, Rosemont, IL, USA), anti-GAPDH (1:2000, Bioss), and β-tubulin (1:3000, Affinity Biosciences). The appropriate horseradish peroxidase-conjugated secondary antibodies were diluted 1:20000 in 1× PBS and added to the membranes for 1 h at room temperature. The protein bands were visualized using enhanced chemiluminescence reagent. ImageJ software was used to analyze the band intensities. β-actin, β-tubulin, and VDAC1 were used as internal controls.

Statistical analysis

All analyses were performed using Prism 7 software (GraphPad, San Diego, CA, USA). When the distribution of data was not normal, a Mann–Whitney U test was used for analysis. Statistical differences of follicle number, litter size, hormone levels and western blot results in four experimental groups were assessed by one-way ANOVA for multiple comparisons followed by Bonferroni *post hoc* analysis. A P-value <0.05 was considered statistically significant.

Results

Mel prevents Cyc-induced primordial follicle loss

To determine whether Cyc activates primordial follicles, Mel has a protective function against Cyc-induced primordial follicle loss. Follicle number and litter size in the mice treated with or without Mel were counted after chemotherapy. Oocytes were stained with Nobox antibody to determine the location of primordial follicles in mouse ovarian tissue. The primordial follicles were labeled (Figure 2A). The number of primordial follicles, primary, secondary, antral, and atretic follicles were counted. After Cyc treatment alone, the number of primordial follicles in the Cyc group was significantly reduced compared to that in the Sal group ($P < 0.05$). In addition, the number of primordial follicles in mice treated with Mel during Cyc chemotherapy (Mel + Cyc group) was significantly higher than that in Cyc-treated mice (Cyc group) ($P < 0.05$).

Compared to the Sal (control) group, Cyc treatment also increased the number of primary follicles, which was significantly reduced by Mel treatment (Figure 2B). Compared to the control group, the average litter size was significantly lower after Cyc chemotherapy on Day 55 (from 115 days after chemotherapy to 170 days after chemotherapy); however, Mel co-treatment significantly increased the average litter size after Cyc treatment (Figures 2C, E). The cumulative pup number of each mouse in the Cyc group was lower than that in the Sal and Mel groups, and Mel co-treatment increased the cumulative number of pups in the Mel + Cyc group (Figure 2D). Overall, Mel treatment prevented Cyc-induced dormant primordial follicle loss and suppressed the conversion of primordial follicles to primary follicles.

Mel significantly improves expression of AMH after Cyc chemotherapy

To determine whether Cyc chemotherapy induced primordial follicle loss through indirect overactivation of primordial follicles and activation of the PTEN/AKT/FOXO3a pathway, FOXO3a phosphorylation levels and serum AMH concentrations were measured. The results revealed that serum AMH and LH levels in the Cyc group decreased significantly 14 days after Cyc chemotherapy, but combination therapy with Mel significantly prevented the Cyc-induced decrease in serum AMH and LH levels in the Mel + Cyc group ($P < 0.05$). Serum FSH levels in the Cyc group were not significantly different from those in the Mel + Cyc group ($P > 0.05$) (Figures 3A, B). Biochemical analysis of ovarian tissue for antioxidant enzymes showed a significant increase in MDA in the Cyc group after Cyc chemotherapy for 14 days, but combined therapy with Mel significantly prevented the Cyc-induced increase in MDA levels in the Mel + Cyc group ($P < 0.05$). Tissue SOD and CAT activities were significantly higher in the Mel + Cyc group than in the Cyc group and lower than those in the Sal and Mel groups in the ovarian homogenates ($P < 0.05$) (Figure 3C). However, no significant changes were observed in the phosphorylation levels of FOXO3a and PTEN (Figure 3D). These results indicate that Cyc chemotherapy over activates primordial follicles through an AMH-mediated indirect pathway, whereas the FOXO3a-mediated pathway does not play a role in this process.

Co-treatment with Mel prevented apoptosis in granulosa cells

To analyze whether Mel maintained AMH expression by inhibiting ovarian granulosa cell apoptosis in growing follicles,

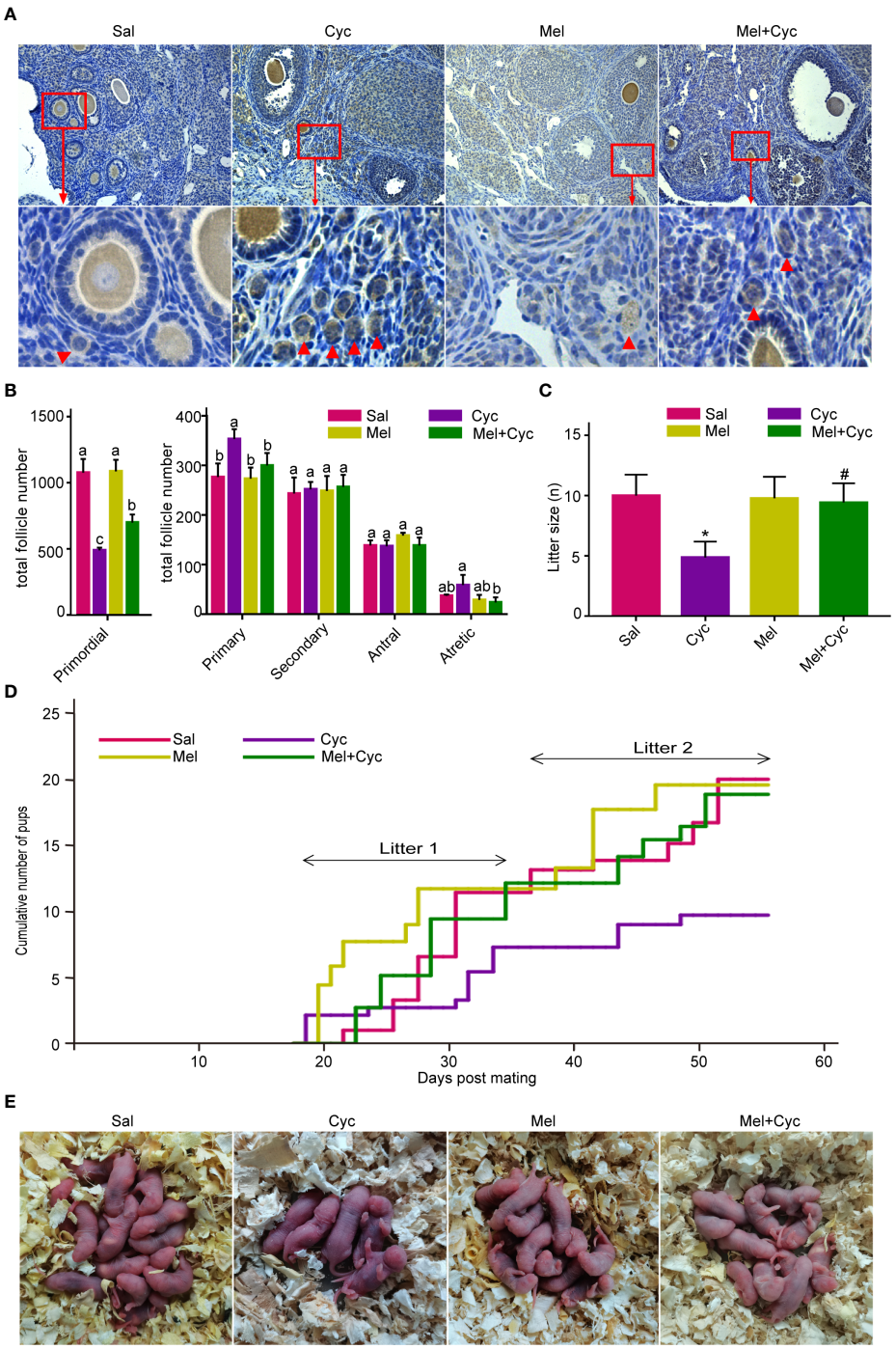

FIGURE 2
Mel prevents Cyc-induced primordial follicle loss. There were 25 six-week-old female mice in each group, of which 18 were sacrificed to obtain the ovary and blood samples, and seven mice were used for the mating experiment. **(A)** Oocytes in mouse ovaries were detected using an Nobox antibody (n = 6). Red arrow indicates primordial follicles. Bar = 200 μm. **(B)** Follicle count of whole ovarian tissue in control and treatment group mice 14 days after Cyc chemotherapy (n = 6). Mel prevented Cyc-induced dormant primordial follicle loss and suppressed the activation of primordial follicles into primary follicles. ^{a,b,c,d} Values with different letters in the same type of follicles were significantly different from each other. **(C)** Cyc treatment reduced the mean litter size in mice and Mel reversed this effect (n = 7). $^{*}P < 0.05$ compared with Sal group. $^{\#}P < 0.05$ compared with Cyc group. **(D)** Cumulative pup number of each mouse in control and treatment groups at Day 55 of mating (n = 7). **(E)** Representative litters from control and treatment groups.

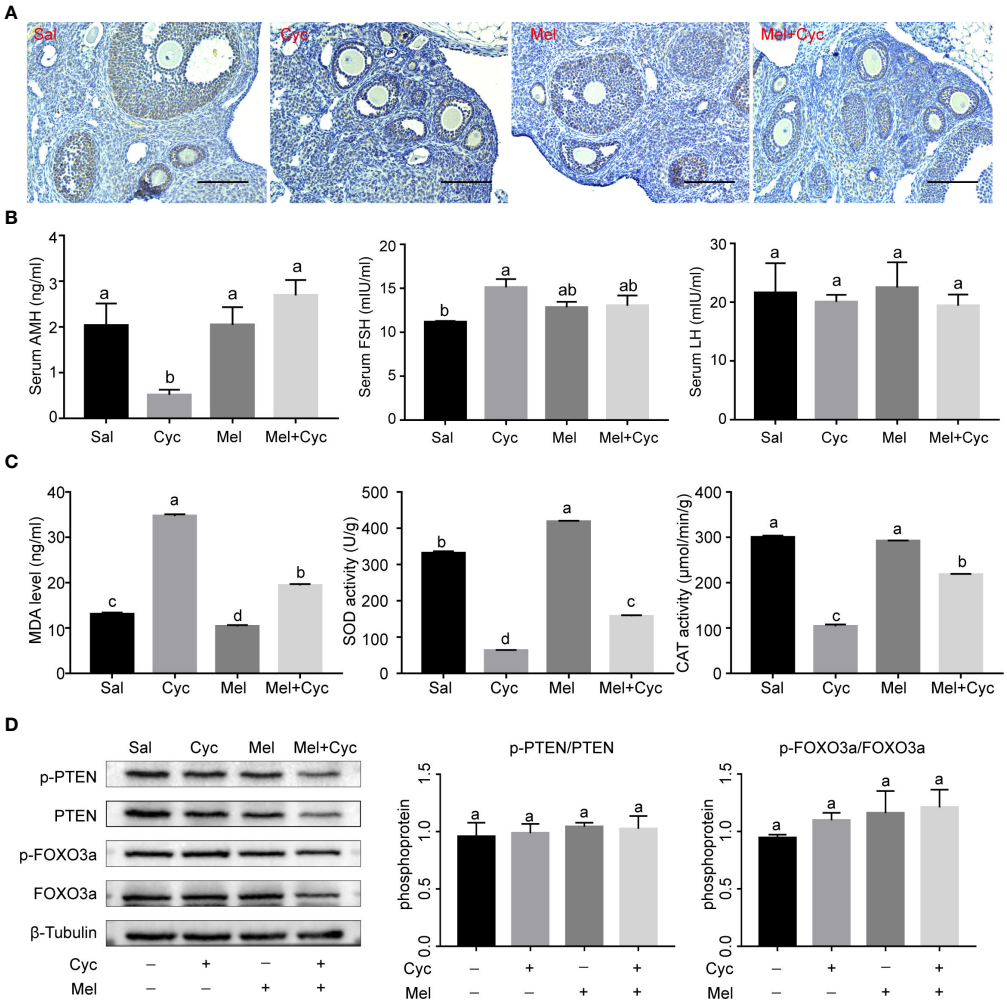

FIGURE 3
Effect of Mel on expression of AMH and oxidative stress in ovaries of six-week-old female mice 14 days after Cyc chemotherapy (Day 18). **(A)** AMH was expressed in granulosa cells of secondary and early antral follicles in Sal, Cyc, Mel, and Mel + Cyc groups (n = 6). Bar = 200 μm. **(B)** Cyc combined with Mel therapy prevented the decrease of serum AMH and LH levels but there was no significant change in serum FSH levels (n = 6). **(C)** Biochemical analysis of the ovarian CAT, SOD activities, and MDA levels (n = 6). **(D)** Phosphorylation levels of PTEN and FOXO3a did not change significantly after treatment with Cyc and Mel (n = 6). ^{a,b} Values with different letters are significantly different from each other.

we measured the incidence of apoptosis of granulosa cells in prepubertal three-week-old female mice after Cyc and/or Mel treatment. Cyc treatment promoted apoptosis of ovarian granulosa cells and the apoptotic peak of granulosa cells was not associated with the injection of PMSG 12 or 24 h in advance but occurred 12 h after Cyc injection (Figures 4A–C). Therefore, we selected 12 h after Cyc injection as the time point to study the effect of Mel intervention on apoptosis of granulosa cells and found that Mel significantly reduced the number of TUNEL-positive apoptotic granulosa cells in growing follicles after Cyc chemotherapy (Figures 4D, E).

Mel inhibits Cyc-induced apoptosis of granulosa cells through mitochondrial apoptosis pathway

To further investigate the inhibitory effect and mechanism of Mel on Cyc-induced apoptosis of granulosa cells, we measured the expression of caspase3, Bax, Bcl-2, and Cyt-C proteins. The results showed that Cyc significantly increased the expression of cleaved-caspase3, Bax, and cytoplasmic Cyt-c and decreased the expression of Bcl-2 in ovaries ($P < 0.05$). Co-treatment with Mel significantly reduced Cyc-induced up-regulated expression of cleaved-caspase3,

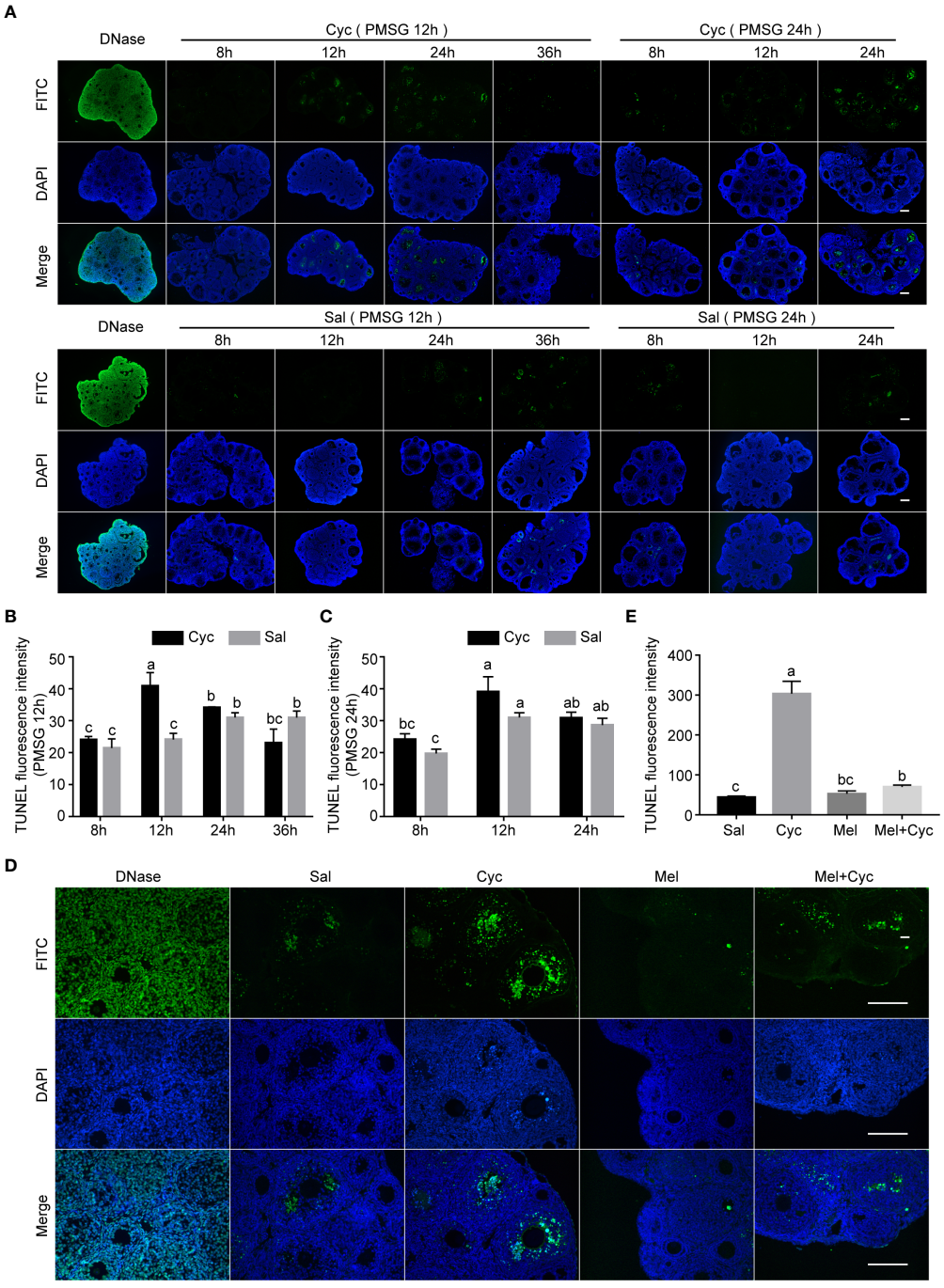

FIGURE 4
Cyc induced apoptosis of granulosa cells in growing follicles and Mel prevented this apoptosis. **(A)** Three-week-old ICR mice were injected i.p. with Cyc 12 or 24 h after injection of PMSG, and apoptosis in ovarian tissues was detected by TUNEL kit at 8, 12, 24, and 36 h after Cyc injection. Regardless of whether Cyc was injected 12 or 24 h after PMSG injection, apoptotic peak of granulosa cells in ovary appeared 12 h after Cyc intervention, which was not related to the time of PMSG administration. Mice in control group were injected with Sal instead of Cyc, and the apoptotic peak of granulosa cells appeared 48 h (PMSG 12 h + Sal 36 h, or PMSG 24 h + Sal 24 h) after injection of PMSG. DNase I treatment was used for positive control of TUNEL assay. There were four three-week-old female mice in each group; Bar = 200 μm. **(B)** TUNEL fluorescence intensity in ovaries of 3-week-old ICR mice 8, 12, 24 and 36 hours after Cyc injection. The mice received Cyc chemotherapy 12 hours after injection of PMSG. **(C)** TUNEL fluorescence intensity in ovaries of 3-week-old ICR mice 8, 12 and 24 hours after Cyc injection. The mice received Cyc chemotherapy 24 hours after injection of PMSG. **(D)** Mel prevented apoptosis of granulosa cells 12 h after Cyc chemotherapy. There were four three-week-old female mice in each group; Bar = 200 μm. **(E)** TUNEL fluorescence intensity in ovaries of 3-week-old ICR mice in Sal, Cyc, Mel and Mel+Cyc groups 12 hours after Cyc injection.

Bax, and cytoplasmic Cyt-c, and increased the Cyc-induced down-regulated expression of Bcl-2 in the ovaries ($P < 0.05$) (Figures 5A, B). These findings suggest that the mitochondrial apoptosis pathway is involved in Cyc-induced apoptosis in granulosa cells, and Mel inhibits the apoptosis pathway.

Discussion

In the present study, we demonstrated that Cyc treatment promoted apoptosis of ovarian granulosa cells through the mitochondrial apoptotic pathway. Apoptosis of granulosa cells reduces AMH secretion and depletes the dormant follicle pool in mouse ovaries through indirect over-activation of primordial follicles. Furthermore, we found that Mel co-treatment significantly prevented Cyc-induced apoptosis of ovarian granulosa cells, which maintained AMH expression in granulosa cells during chemotherapy, and prevented Cyc-induced primordial follicle loss in mouse ovaries.

Cyc is a chemotherapeutic drug that is highly toxic to ovaries. It is a prodrug that is activated by cytochrome p450 enzymes to produce its active metabolites. The latter is responsible for ovarian toxicity. Cyc does not induce degeneration of primordial follicles. Instead, Cyc induces apoptosis of actively growing follicles and activates primordial follicles to primary follicles, leading to the depletion of primordial follicles (8). Cyc induces apoptosis through two pathways, one of which involves Cyc inhibition of the synthesis of DNA by cross-linking with cell DNA in all stages of the cell cycle, causing cellular apoptosis (10, 31). The other pathway involves the reduction of mitochondrial transmembrane potential caused by Cyc and accumulation of Cyt-c in the cytosol of rat granulosa cells, which leads to the activation of the caspase family and apoptosis (11). Cyc also upregulates the expression of Bax protein and downregulates the

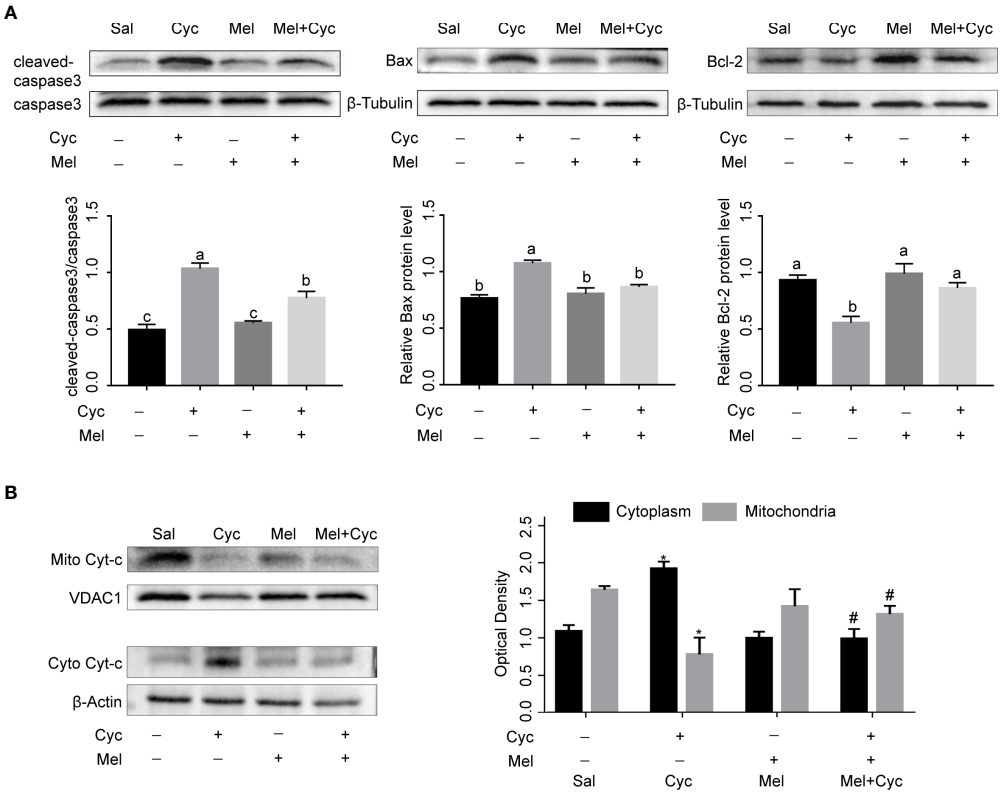

FIGURE 5
Mel inhibits Cyc-induced apoptosis of granulosa cells through mitochondrial apoptosis pathway. **(A)** Western blot analysis was performed on cleaved-caspase3, caspase3, Bax, and Bcl-2 in the ovaries of three-week-old ICR mice 12 h after Cyc (75 mg/kg) treatment with or without Mel (n = 6). ^{a,b,c} Bars with different letters are significantly different in each group ($P < 0.05$). **(B)** Western blot analysis was performed on mitochondrial Cyt-c (mito Cyt-c) and cytoplasm Cyt-c (cyto Cyt-c) in the ovaries of three-week-old ICR mice 12 h after Cyc (75 mg/kg) treatment with or without Mel (n = 6). *$P < 0.05$ compared with Sal group. $^{\#}P < 0.05$ compared with Cyc group.

expression of Bcl-2 which causes the reduction of mitochondrial transmembrane potential, causing activation of the apoptotic cascade (11). Our results suggest that Cyc stimulates apoptosis in growing follicular granule cells through a mitochondria-dependent pathway in mice. The apoptosis of granulosa cells reduces AMH secretion and depletes the dormant follicle pool in mouse ovaries through indirect over-activation of primordial follicles. However, the addition of Mel inhibited apoptosis of granulosa cells induced by Cyc and maintained AMH expression in the ovaries.

Physiologically, apoptosis is an essential event for ovarian function, and the development of this organ is harmful when the ovary is exposed to Cyc (3, 32). The Cyc drug itself and its toxic metabolites also interfere with the intracellular antioxidant system, which plays an important function in detoxifying reactive oxygen species (33). SOD can transform superoxide anion into hydrogen peroxide, which plays a key role in antioxidant reaction (34, 35). CAT, another antioxidant enzyme, catalyzes only the decomposition of hydrogen peroxide into water and oxygen in the absence of an electron donor (34, 36). Biochemical measurements of MDA levels in tissues as a measure of lipid peroxidation have been used to assess oxidative stress and ovarian damage (35–37). The results of previous research indicate that Cyc decreases SOD and CAT activity, which means that the consumption of this antioxidant enzyme is increased by Cyc or its metabolites. In the present study, the parameters of oxidative stress, that is MDA, were significantly increased and the activities of SOD and CAT were significantly decreased in the ovaries of Cyc-treated mice, suggesting that Cyc treatment caused oxidative damage to lipids and proteins in this organ. The mouse ovaries in the Mel + Cyc group had significantly increased SOD and CAT activities and decreased MDA levels, suggesting that Mel protects against the adverse effects of Cyc.

Mel is a powerful free-radical scavenger and broad-spectrum antioxidant (38, 39). Mel has several important protective and regulatory functions. In addition to its role in regulating sleep, Mel also protects ovarian function, regulates immune function, and has anti-aging and anti-tumor (40–42). In the female reproductive system, Mel plays an important role in normal physiology and protects against ovarian pathologies (21, 43). Mel, a free radical scavenger in ovarian follicles, promotes oocyte maturation, embryo development, and luteinization of granulosa cells (44, 45). In the current study, we found that Mel plays an antioxidative role by reducing MDA content and increasing SOD and CAT activity. We also found that Mel ameliorates the effects of mitochondria-mediated apoptosis on ovarian tissue by inhibiting Bax expression, Cyt-c release from the mitochondria to the cytoplasm, caspase-3 activation, and induction of Bcl-2 expression. Mel protects ovarian granulosa cells from apoptosis induced by Cyc chemotherapy by inhibiting the mitochondrial apoptosis pathway, providing new evidence for Mel as an adjuvant chemotherapy agent.

AMH, which is secreted by the granulosa cells of growing follicles, represents a reliable biomedical marker of ovarian reserve and is informative for monitoring ovarian function after chemotherapy (46, 47). AMH level is an important indicator of ovarian function after chemotherapy. A rapid and significant decrease in AMH concentration has been observed in adult women after chemotherapy (48). Recent data also suggest that the AMH concentration before chemotherapy and the size of the drop and recovery of AMH during and after chemotherapy can be used to predict the degree of ovarian injury (49). Therefore, the use of adjuvant drugs to protect AMH levels during chemotherapy is particularly important for maintaining fertility in women treated with antitumor chemotherapy. When injected or overexpressed in mice concurrent with chemotherapy, AMH inhibits the activation of primordial follicles and prevents POF (12). Prevention of POF and protection of the ovarian follicle pool have become attractive avenues for improving the quality of life of female cancer patients receiving chemotherapy. Fertility preservation in female patients is important for their well-being after cancer survival. In the current study, Mel treatment significantly reversed the decrease in serum AMH levels, possibly because Mel intervention prevented Cyc-induced apoptosis of the follicular granulosa cells. The surviving granulosa cells secrete AMH and maintain normal serum AMH levels. AMH inhibits over-activation of primordial follicles through negative feedback regulation and prevents Cyc-induced primordial follicle loss.

Litter size is one of the methods used for the functional evaluation of the ovarian reserve after chemotherapy. primordial follicles generally require many weeks to develop into mature follicles in mice (50). In the present study, to ensure that mature oocytes were derived from primordial follicles after chemotherapy, mice were selected 115 days after Cyc treatment for two cycles of mating, and we found that Cyc significantly reduced the average litter size in mice. In the Mel intervention group, litter size increased significantly after chemotherapy. Apoptosis of granulosa cells induced by Cyc chemotherapy was reduced after intervention with Mel, and the surviving granulosa cells maintained the expression of AMH, thus inhibiting chemotherapy-induced hyperactivation of primordial follicles. In addition, a recent study showed that Cyc induces primordial follicle loss through the PTEN/AKT/FOXO3a signaling pathway (51); however, we showed here that Cyc had no effect on the PTEN/AKT/FOXO3a pathway. This may be related to the dose of Cyc, which was 200 mg/kg in the study performed by Barberino compared to 75 mg/kg used in the present study.

Conclusion

In summary, our findings suggest that Mel prevents Cyc-induced over-activation of primordial follicles by inhibiting ovarian granulosa cell apoptosis and maintaining AMH expression. Cyc exposure disturbs the balance of follicle activation by increasing apoptosis of AMH-secreting granulosa cells in growing follicles, resulting in the further decrease of AMH levels and indirect over-activation of the primordial follicle pool. The activated follicles undergo apoptosis, and more primordial follicles are stimulated to become activated primary follicles, resulting in POF. However, Mel co-treatment reduced primordial follicle activation and apoptosis of AMH-secreting granulosa cells in growing follicles. Mel restored the balance of follicle activation and returned the ovary to a healthy state (Figure 6). These findings on the mechanisms underlying the protective effects of Mel against Cyc-induced primordial follicle loss have key implications for fertility maintenance in young cancer patients who undergo chemotherapy.

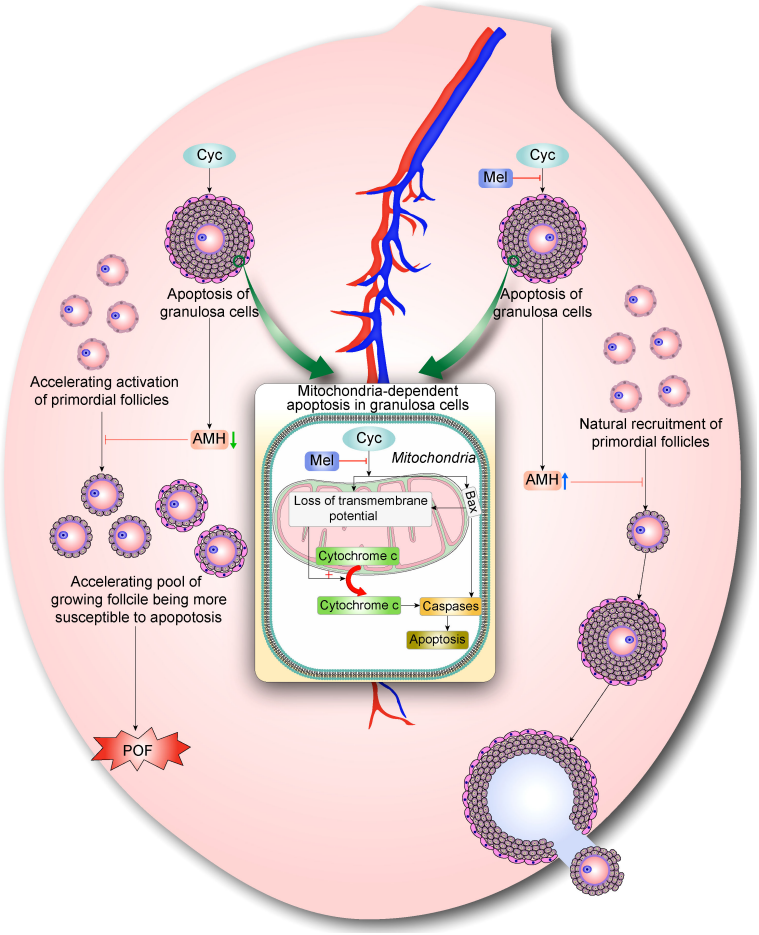

FIGURE 6
Mel prevents Cyc-induced over-activation of primordial follicles through inhibiting ovarian granulosa cell apoptosis and maintaining AMH expression. During normal follicle development, very few primordial follicles are selected and activated, whereas the vast majority of primordial follicles are maintained in a dormant state. AMH is mainly produced by granulosa cells in secondary follicles and early antral follicles. AMH can inhibit the activation of primordial follicles during recruitment and inhibit the sensitivity of antral follicles to FSH during recruitment cycle, thus maintaining AMH levels and selective activation of primordial follicle pool. Cyc exposure disturbs the balance of follicle activation by increasing apoptosis of AMH-secreting granulosa cells in growing follicles, resulting in further decrease of AMH levels and indirect over-activation of primordial follicle pool. Eventually, activated follicles undergo apoptosis and more primordial follicles are stimulated to become activated primary follicles, resulting in POF. With Mel co-treatment, activation of primordial follicles is reduced and apoptosis of AMH-secreting granulosa cells in growing follicles is decreased. Surviving granulosa cells maintained normal levels of AMH production, which regulates natural recruitment of primordial follicles and oogenesis. Hence, Mel restores the balance of follicle activation and returns ovaries to a healthy state.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animals Care and Use Committee of Ningxia Medical University.

Author contributions

JF, W-WM, and H-XL were responsible for the experiments, data analysis and editing of the manuscript. X-YP, S-LD, and HJ participated in the design of the study and edited the manuscript. W-ZM contributed to the conception, supervision and editing of the manuscript. All authors read and approved the final manuscript.

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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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EDITED BY
Mahmoud Salama,
Michigan State University,
United States

REVIEWED BY
Christine Wyns,
Catholic University of Louvain, Belgium
Alexandre Rodrigues Silva,
Federal University Rural
Semi-Arid, Brazil

*CORRESPONDENCE
Dehlia Moussaoui
dehlia.moussaoui@hcuge.ch

†PRESENT ADDRESS
Dehlia Moussaoui,
Department of Pediatric and
Adolescent Gynecology, The Royal
Children's Hospital Melbourne,
Parkville, VIC, Australia

‡These authors have contributed equally to this work and share first authorship
§These authors have contributed equally to this work and share last authorship

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Testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys: A 6 year experience from a Swiss multi-center network

Dehlia Moussaoui[1*†‡], Anna Surbone[2‡], Cécile Adam[3], Tamara Diesch-Furlanetto[4], Céline Girardin[5], Julie Bénard[6], Isabelle Vidal[7], Fanette Bernard[8,9], Kanete Busiah[10], Thérèse Bouthors[10], Marie-Pierre Primi[11], Marc Ansari[8,9], Nicolas Vulliemoz[2§] and Fabienne Gumy-Pause[8,9§] on behalf of the HUG-CHUV-UKBB Fertility Preservation Pediatric Group

[1]Division of General Pediatrics, Department of Woman, Child and Adolescent Medicine, Geneva University Hospitals, Geneva, Switzerland, [2]Fertility Medicine and Gynaecologic Endocrinology Unit, Department Woman-Mother-Child, Lausanne University Hospital, Lausanne, Switzerland, [3]Oncology and Hematology Unit, Service of Pediatrics, Department Woman-Mother-Child, Lausanne University Hospital, Lausanne, Switzerland, [4]Division of Pediatric Oncology-Hematology, University Children's Hospital of Basel, Basel, Switzerland, [5]Pediatric Endocrine and Diabetes Unit, Department of Woman, Child and Adolescent Medicine, Geneva University Hospitals, Geneva, Switzerland, [6]Unit for Reproductive Medicine and Gynecological Endocrinology, Department of Woman, Child and Adolescent Medicine, Geneva University Hospitals, Geneva, Switzerland, [7]Division of Pediatric Surgery, Department of Woman, Child and Adolescent Medicine, University Center of Pediatric Surgery of Western Switzerland, Geneva University Hospitals, Geneva, Switzerland, [8]Pediatric Oncology and Hematology Unit, Department of Women Child and Adolescent, University Hospitals of Geneva, Geneva, Switzerland, [9]CANSEARCH Research Platform for Pediatric Oncology and Hematology, Department of Pediatrics, Gynecology and Obstetrics, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [10]Pediatric Endocrinology, Diabetology and Obesity Unit, Service of Pediatrics, Department Woman-Mother-Child, Lausanne University Hospital, Lausanne, Switzerland, [11]Laboratory of Andrology and Reproductive Biology, Department Woman-Mother-Child, Lausanne University Hospital, Lausanne, Switzerland

Testicular tissue cryopreservation is the only option of fertility preservation in prepubertal boys. While it is considered experimental, since procedures to obtain mature spermatozoa from prepubertal testicular tissue are still under development, testicular tissue cryopreservation programs have emerged worldwide. Our aim was to study the feasibility and safety of a program of testicular tissue cryopreservation in prepubertal and adolescent boys facing gonadotoxic treatment in three University hospitals in Switzerland. Testicular tissue cryopreservation was accepted by 90% of families, with a total of 35 patients included. The average patient age was 8.5 years (range 7 months to 18.5 years). Malignancies were the most common diagnosis (31 patients, 88.6%) with 16 (45.7%) solid tumors and 15 (42.9%) hematological malignancies. Four (11.4%) patients had a benign condition. The main indication for testicular tissue cryopreservation was conditioning for

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hematologic stem cell transplantation (25 patients, 71.4%). Testicular tissue was cryopreserved according to the freezing protocol of Louvain Catholic University (Belgium), which includes either only immature testicular tissue freezing, or mature and immature testicular tissue freezing depending on the age of the patient and the presence or absence of haploid cells. The median number of spermatogonia per tubule cross-section was 2 (range 0–6) and spermatozoa were found in only one patient. Tumoral cells were found in one testicular biopsy of a leukemic patient. There were two minor adverse events and none of them required medical treatment or surgical revision. Five patients died during follow-up. Our data demonstrate the feasibility and safety of a program of testicular tissue cryopreservation coordinated by a multidisciplinary team of fertility preservation. Despite the experimental aspect of the procedure, the acceptation rate was high, which highlights the willingness of families and patients to participate in testicular tissue cryopreservation.

KEYWORDS

testicular tissue cryopreservation, fertility preservation, prepubertal boys, oncology, gonadotoxicity

Introduction

The development of oncologic treatments has allowed for significant improvement of life expectancy and survival in children diagnosed with cancer (1). The survivors of these oncologic therapies, however, can experience long-term side effects including infertility caused by impaired spermatozoa production including azoospermia (2–4). These well-described long-term effects are related to the gonadotoxic oncologic treatments such as chemotherapy and localized radiotherapy. In prepubertal and adolescent boys, where mature spermatozoa cannot be cryopreserved, the only option for fertility preservation in cases requiring gonadotoxic therapy is testicular tissue cryopreservation (TTC) (5). The goal of TTC is to preserve spermatogonial stem cells. Although this technique is considered experimental as it has not yet been possible to produce mature spermatozoa (with reproductive potential) from human spermatogonia, the recent birth of a female non-human primate following autografting of cryopreserved immature testicular tissue represents a major step to support TTC in prepubertal and adolescent boys (6).

TTC in boys has been discussed for more than 20 years but remains an ethical and legal challenge (7). Fertility preservation programs in prepubertal boys have emerged worldwide but data regarding the outcomes are still limited. In this article, we present 6 years of experience of fertility preservation in prepubertal and adolescent boys in a multi-center network.

Materials and methods

Patients and study design

Data were prospectively collected from patients between 0 and 19 years of age who underwent TTC at the Lausanne University Hospital (CHUV), Geneva University Hospitals (HUG), and Basel University Children's Hospital (UKBB) in Switzerland between 2015 and 2020. The indication for TTC was reviewed by a multidisciplinary team dedicated to fertility preservation and the procedure was offered to the family after reaching consensus. Written informed consent was obtained from the guardian(s) prior to inclusion in the study or from the patient if deemed to have consent capacity.

Eligibility criteria are described in Table 1. Chemotherapeutic agents were classified in high and respectively low gonadotoxic drugs, based on the recommendations of the Oncofertility consortium (8), CECOS (Centre d'étude et de conservation des oeufs et du sperme) (9) and on the literature (10–14). Collected data included patient age, pubertal development according to Tanner stages, diagnosis, indication for TTC, and previous exposure to chemotherapy and radiotherapy. The Tanner stage was evaluated by the pediatrician in charge of the child or by the endocrinologist using the pubic hair staging (15). Alkylating chemotherapy exposure was calculated using the cyclophosphamide equivalent dose (CED) calculator (11). The amount of collected testicular tissue, adverse events and living status were reported.

TABLE 1 Inclusion and exclusion criteria for testicular tissue cryopreservation.

Inclusion criteria
Prepubertal boys (Tanner 1) greater than 3 months of age
Peri and post-pubertal boys (Tanner 2–4 and Tanner 5 respectively) with unsuccessful or impossible cryopreservation of mature sperm
Scheduled to undergo high-risk gonadotoxic treatment such as:
• High dose alkylating chemotherapy
• High dose cisplatine
• Testicular radiation
• Total body irradiation
Consensus of the multidisciplinary team dedicated to fertility preservation
Exclusion criteria
Less than 3 months of age
Guardian or patient refusal
Non high-risk gonadotoxic treatment

Chemotherapeutic agents were classified in high respectively low gonadotoxic drugs, based on the recommendations of the Oncofertility consortium (8), CECOS (Centre d'étude et de conservation des oeufs et du sperme) (9) and on the literature (10–14).

Tissue retrieval, transportation, and cryopreservation

Testicular tissue was obtained by a unilateral open testicular biopsy performed by a trained, pediatric surgeon under general anesthesia. Whenever possible, the testicular biopsy was performed at the same time as another surgical procedure requiring general anesthesia. Less than one third of the unilateral testicular volume was retrieved. Testicular tissue samples were transferred to Falcon tube (50 ml, Ref. 352098) containing a phosphate-buffered solution (PBS) at 4°C and transported on ice to the Laboratory of Andrology and Reproductive Biology (LABR) of Lausanne University Hospital, which centralized all samples from the three centers. The maximum transport time was 4 hours. There was no temperature monitoring during transport.

For boys younger than 10 years old, testicular tissue was cryopreserved according to the immature testicular tissue freezing protocol of Louvain Catholic University, Belgium (16). On arrival at the laboratory, in no more than 10 min, the biopsy was transferred in a Petri dish positioned on ice (4°C), was divided in 1–2 mm^3 fragments, which were then placed in cryotubes containing 1 ml of the cryoprotectant solution (Sucrose 0.1 ml/l, DMSO 0.7 mol/l, HSA10 mg/ml). Once the cryotubes were sealed (SYMS III, Cryo Bio System, France), the slow freezing was performed using a programmable freezer (FREEZAL, Air Liquide, Carbagas, Suisse). At the end of the freezing program, the cryotubes were stored at −196°C in liquid nitrogen. A fragment of the extracted tissue was fixed in Formaldehyde 10% and sent to pathology department for histological examination on Haematoxylin-Eosin stained slides. Additional immunohistochemical staining was performed to assess the presence of spermatogonia using specific markers (SALL4 and CD117) and to detect tumoral cells (antibodies according to the underlying disease). Spermatogonia counting was performed per tubule cross-section. In average, 20 seminiferous tubule cross sections were counted. For boys above the age of 10, the method of cryopreservation was defined after tissue analysis according to the protocol of Louvain Catholic University: if haploid cells were observed, half of the sample was cryopreserved according to the mature testicular tissue freezing protocol (17), and the other half according to the immature testicular tissue freezing protocol to increase the chance of subsequent fertility restoration. The mature testicular tissue freezing protocol consists of the mincing of the tissue in a Petri dish and decantation of the solution with G-MOPS-PLUS for 10 minutes. The cell suspension is placed in a first tube. The supernatant is removed, placed in a second tube and centrifugated at 300 g for 10 min. The supernatant is disposed and the cell suspension from the first and the second tubes are mixed. A droplet of this suspension is aspirated and evaluated for spermatozoa counting. An equal amount of freezing medium (Irvine Scientific, No. 9971) is added to the cell suspension and aliquots of 0.5ml are transferred into high security straws (CryoBioSystem, France) which are then sealed in both ends. The straws are placed in a programmable freezer (FREEZAL, Air Liquide, Carbagas, Suisse), which gradually lowers the temperature from 20 to −150°C, and then transferred to cryotanks filled with liquid nitrogen for storage at −196°C. If no mature cells were observed, mature and immature testicular tissue freezing was only completed for boys older than 12 and only immature testicular tissue freezing was done for boys younger than 12.

Apart from the fragment sent to pathology, all other fragments were destined for future clinical use. In case of death, the tissue was either destroyed or conserved in an anonymized fashion for research purposes if the consenting individual(s) had signed the corresponding consent.

Statistics

Statistical analyses were performed using STATA software (version 16.0). Mean, median and percentages were calculated to describe patient characteristics, indications for fertility preservation, treatment exposure before testicular cryopreservation, and the amount of collected testicular tissue. The Mann-Whitney U test was used for the comparison of spermatogonia count. A two-sided *P*-value of <0.05 was considered to be significant.

Study approval/ethics

This study was approved by the local ethics committee (PB_2016-01378) and registered with clinicaltrials.gov (NCT03180918). Each center's protocol was also approved by their respective Institutional Review Board.

Results

TTC was indicated and offered to 40 patients. Four families declined the procedure. In one case, TTC was accepted by the parents but not completed due to urgency of hematologic stem cell transplantation (HSCT).

Testicular tissue from 35 patients was collected and cryopreserved between April 2015 and September 2020 (Table 2). During the collection period we observed a progressive increase in the number of TTC procedures with the exception of 2020 (3 in 2015, 3 in 2016, 4 in 2017, 8 in 2018, 11 in 2019 and 6 in 2020).

The mean patient age was 8.5 years (SD 5.1) and ranged from 7 months to 18.5 years. Twenty-four (69%) patients were prepubertal (Tanner 1), while 9 (26%) were on ongoing puberty (Tanner 2-4). Two boys (5%) with completed puberty (Tanner 5) underwent TTC due to the inability to provide a semen sample by masturbation. Underlying diagnoses requiring gonadotoxic therapy were a malignant disorder in 31 patients including 15 (42.9%) hematological malignancies and 16 (45.7%) solid tumors (Figure 1). Four (11.4%) patients had a benign condition. The primary indication for TTC was conditioning for HSCT (25 patients, 71.4%). Among patients with solid tumors, 7 underwent TTC because of gonadotoxic chemotherapy and radiation (3 medulloblastoma, 1 germ cell tumor, 2 rhabdomyosarcoma, and 1 Ewing sarcoma), 2 because of high dose cisplatine (2 osteosarcoma), and one because of testicular radiation (nephroblastoma stage IV). Nineteen patients (54.3%) had already been exposed to chemotherapy before testicular biopsy, including 16 (45.7%) to alkylating chemotherapy. Average previous CED exposure was 5,466 mg/m^2 (SD 3,362, range 2,000–15,576 mg/m^2).

In 23 patients (65.7%), the testicular biopsy was performed at the same time as another surgical procedure requiring general anesthesia. Adverse events were rare: one patient suffered from a minor hematoma and another from a minor wound dehiscence. None of them required medical treatment or surgical revision. The number of testicular tissue fragments varied throughout our series with a median of 29 fragments (range 12–60), corresponding to a median volume of the testicular biopsy of 57 mm^3 (range 24–120 mm^3). The median number of spermatogonia per tubule cross-section was 2 (range 0–6). In patients having received alkylating chemotherapy prior to TTC, the median number of spermatogonia was significantly lower than in patients who had not yet received alkylating chemotherapy (0.5 with a range 0–4, and 2.75 with a range 0–6 respectively, $p = 0.0017$). Spermatozoa were found in one patient, aged 15 and who had not received any prior chemotherapy. Based on histology and immunohistochemistry, tumoral cells were found in one testicular biopsy of a leukemic infant. This patient was diagnosed with B-ALL MLL+ at the age of 5 months and treated according to INTERFANT-06 protocol. He was in complete remission with negative bone marrow minimal residual disease (BM-MRD) at the end of induction. However, at the start of MARMA phase, cerebrospinal fluid showed blasts and central nervous system treatment was reinforced before HSCT. BM-MRD was negative before HSCT. The results of the testicular biopsy came after the HSCT and showed leukemic infiltration although he had no clinical sign of testicular involvement. Bilateral testicular biopsies were performed 1 month after HSCT showing no leukemic cells at the immunohistochemical evaluation.

During follow-up five patients died due to tumor progression. Testicular tissue was destroyed in two cases and preserved in three cases, according to the preferences indicated at the time of consent.

Discussion

This prospective study describes 6 years of experience with pre-pubertal and pubertal TTC. Data from 35 patients was reported, which represents a large prospective series on pre-pubertal and pubertal TTC.

Testicular biopsies were performed in 3 Swiss university hospitals after review by a multidisciplinary team dedicated to fertility preservation. The multicenter design allows for generalization of the findings as well as operator-dependent outcomes (such as complication rate or sample quality) are limited. Similar to other studies, pediatric surgeons removed <1/3 of the entire testicular volume (18, 19).

A single laboratory performed all freezing procedures using a well-validated protocol thus limiting variability in sample handling (20). The centralization of all cryopreservation procedures in a single laboratory could, however, raise concerns regarding sample stability and the optimal timing between surgery and biopsy cryopreservation. As all samples were immediately stored at 4°C in a phosphate-buffered medium, the time elapsed between surgery and tissue manipulation at LABR should have not affected the biopsy quality, as demonstrated by Faes and Goossens in 2016 (21). In their study, testicular tissue could be preserved up to 3 days at 4°C without altering the characteristics of gonadal and somatic cells.

Currently, in prepubertal boys, the only option for fertility preservation is immature testicular tissue biopsy. The present study includes malignant (22) and benign (4) conditions, all requiring a highly gonadotoxic treatment. Interestingly, during the study period, we observed an exponential increase in

TABLE 2 Patient characteristics, indication for testicular tissue cryopreservation (TTC), treatment received before TTC, amount of retrieved tissue, and clinical complications. CED exposure was based on the cyclophosphamide equivalent dose calculator (11).

	Patient characteristic			Indication to fertility preservation					Treatment received before testicular tissue cryopreservation				Testicular tissue cryopreservation			
Diagnosis	**Number of patients, *n* (%)**	**Age (y), mean (SD, range)**	**Prepubertal[c] *n* (%)**	**Conditioning for HSCT, chemotherapy alone, *n* (%)**	**Conditioning for HSCT, chemotherapy and radiotherapy, *n* (%)**	**High dose chemotherapy, *n* (%)**	**Local radiotherapy, *n* (%)**	**Expected CED exposure (mg/m^2), mean (SD, range)**	**Previous exposure to chemotherapy, *n* (%)**	**Previous exposure to alkylating chemotherapy, *n* (%)**	**Previous exposure to radiotherapy, *n* (%)**	**Previous CED exposure (mg/m^2), mean (SD, range)**	**Number of testicular tissue fragments, median (SD)**	**Volume of testicular tissue biopsy (mm^3), median (range)**	**Number of spermatogonia/cross section, median (range)**	**Clinical complications**
Malignancies	31 (88.6)	8.6 (5.3, 0.5–18.5)	21 (67.7)	14 (45.2)	7 (22.6)	9 (29)	1 (3.2)	13,002 (11,756, 100–61,200)	19 (61.3)	16 (51.6)	2 (6.5)[b]	5,466 (3,362, 2,000–15,576)	27 (12–60)	54 (24–120)	2 (0–6)	1 minor hematoma
Hematological malignancies	15 (42.9)	10.4 (5.6, 0.7–18.5)	8 (53.3)	8 (53.3)	7 (46.7)	0	0	8,365 (5,403, 100–18,388)	13 (86.7)	11 (73.3)	0 (0)	4,342 (1,394, 2,000–7,400)	39 (16–60)	78 (32–120)	1 (0–6)	1 minor hematoma
Solid Tumors	16 (45.7)	6.8 (4.5, 0.5–14.7)	13 (81.3)	6 (37.5)	0	9 (56.3)	1 (6.3)	16,212 (14,322, 8,892–61,200)	6 (37.5)	5 (31.3)	2 (12.5)[b]	7,940 (4,906, 3,125–15,576)	26 (12–55)	52 (24–110)	2.5 (0–5)	0
Benign conditions[a]	4 (11.4)	7.9 (2.9, 5.5–12)	3 (75)	4 (100)	0	0	0 (0)	1,1012 (5,446, 9,388–12,000)	0 (0)	0 (0)	0 (0)	0 (0)	31 (19–40)	62 (38–80)	2.5 (1–3.5)	1 minor wound dehiscence
Total	35 (100)	8.5 (5.1, 0.5–18.5)	24 (68.6)	18 (51.4)	7 (20)	9 (25.7)	1 (2.9)	12,696 (11,059, 100–61,200)	19 (54.3)	16 (45.7)	2 (5.7)	5,466 (3,362, 2,000–15,576)	29 (12–60)	57 (24–120)	2 (0–6)	2

[a]Sickle cell disease and thalassemia.
[b]Cranio-spinal irradiation (medulloblastoma).
[c]Tanner 1.
HSCT, hematopoietic stem cell transplantation.
CED, Cyclophosphamide equivalent dose.

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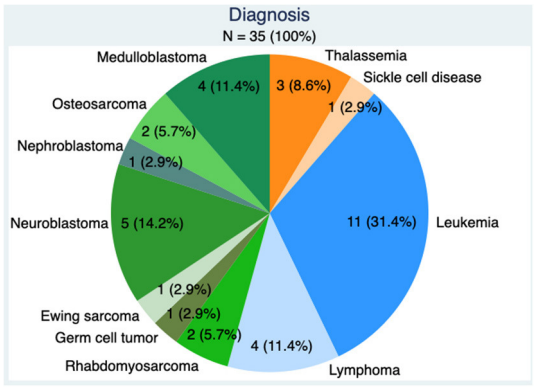

FIGURE 1
Diagnosis in the 35 patients, benign conditions in orange, hematological malignancies in blue, and solid tumors in green.

the number of TTC procedures, with the exception of 2020. This demonstrates successful implementation of an efficient fertility preservation program in boys at the university hospitals involved in the study. The reduction of cases in 2020 might be related to the cancellation of non-urgent HSCT in the context of the COVID-19 pandemic or to fluctuations over time.

Fertility preservation by human immature testicular tissue biopsy is, at present, still experimental as immature testicular tissue was not used *in vitro* or *in vivo* (after grafting) to produce mature spermatozoa with a real reproductive potential.

The easiest option for immature testicular tissue use is by autografting. In patients with malignant disease, examination by molecular techniques of the tissue is mandatory to exclude reintroduction of malignant cells, whereas in non-malignant diseases like hemoglobinopathies there is no restriction for transplantation of the tissue. However, methods for detection of minimal residual disease as multicolor flow cytometry, RT-qPCR, next-generation sequencing and xenograft of tissue in immunodeficient mice are still in development in this context, mainly in ovarian tissue preservation, and validated strategies to ensure the complete safety are still lacking (23–25). A birth *via* autografting has been achieved in non-human primates, indicating the potential feasibility of this technique in humans (6). *In vitro* production of spermatozoa would nevertheless represent the ideal approach as it would avoid the potential risk of reimplantation of tumoral cells associated with autografting. *In vitro* production of spermatozoa is the focus of intense research with several steps successfully realized in the recent years (26). A study published in 2018 demonstrated the feasibility of generating haploid germ cells from immature testicular tissue in organotypic cells cultures, but the real reproductive potential of these cells is still to be demonstrated (27). Another option to avoid the risk of cancer cells contamination is the *in vivo* development of testicular organoids, which allows for prior cell selection. Different studies have recently reported the successful development of testicular function units in rats and pigs (28–31). Research is still ongoing to evaluate the ideal culture medium and conditions necessary to obtain sustained testicular architecture and function (22).

All but four families consented to testicular tissue biopsy, which represents an acceptance rate of 90%, in alignment with what has been reported in the literature (32–34). The decline reason for the four patients was not documented, but the experimental aspect of the procedure may have played a role. To increase parental acceptance and because the procedure is still experimental, the testicular biopsy was coordinated, when possible, with another surgical procedure, to avoid additional exposure to anesthesia and surgical risks for the sole purpose of fertility preservation.

In this series, only a few minor complications occurred (2/35 = 5.7%), demonstrating the safety of this procedure. This finding aligns with previous reports, including larger series (Kanbar et al. reported a complication rate of 3/139 = 2.1%) (20).

One theoretical concern is the presence of tumoral cells within the testicular tissue. This is of special concern in malignancies with a high rate of cancer cell dissemination, for example in leukemia, which represents the majority of the malignancies in our series. A retrospective study found a rate of malignant cells contamination in the testis of boys affected by acute lymphoblastic leukemia as high as 30% (35). Despite this, in the present study, tumoral cell contamination of testicular tissue was found on histology and immunohistochemistry in only one case (a case of acute lymphoblastic leukemia with MLL rearrangement), even though the patient had already been treated by chemotherapy prior to the fertility preservation procedure. Nevertheless, the detection sensitivity of tumoral cells in testicular tissue could be certainly improved by using molecular techniques similar to those used for the quantification of the minimal residual disease.

In our series, 61.3% of the patients with a malignancy had already been treated by chemotherapy at the time of testicular tissue cryopreservation, with 51.6% having received an alkylating chemotherapy at an average CED (cyclophosphamide equivalent dose) of 5,466 mg/m^2 (range 2,000–15,576). This finding contrasts with the results presented by Kanbar et al., where only 7% of the patients had already received a gonadotoxic therapy prior to the fertility preservation procedure (20). On the contrary, in 2019 Valli-Pulaski et al. described the results of their eight-year experience with pre-pubertal boys fertility preservation programs in several recruitment centers in USA and abroad (19). In their series, 39% of the children had already received a gonadotoxic treatment at the time of testicular tissue biopsy, although at a lower mean dose than in our study (average CED = 2,821 mg/m^2, range 500–7,000).

Although only based on histology and immunohistochemistry and not on molecular biology, the

low rate of tumoral cell contamination of testicular tissue in our series may be due to the high proportion of patients having received a prior chemotherapy, as suggested by Borgström et al. in a recent paper (18). In their series of 21 prepubertal boys undergoing TTC prior to HSCT, including 20 patients with a malignant disease, of which 10 with a leukemia, histopathological analysis found leukemia cells in only one patient. In their opinion, the best time for testicular biopsy in acute lymphocytic leukemia is just before HSCT, when circulating blasts have already been eliminated by the previous chemotherapy. Notwithstanding this, in our series, the only patient with tumoral cell contamination of the testicular sample had previously received chemotherapy.

The real impact of previous chemotherapy on immature testicular tissue sampling, and, in particular, on the quality and number of spermatogonia, is still undefined, even though CED above 4,000 mg/m^2 could potentially impact spermatogenesis (10). Our study showed a statistically significant difference in the number of spermatogonia according to the prior exposure to alkylating chemotherapy. In the study published by Stukenborg et al., the spermatogonia number per transverse tubular cross-section was significantly reduced in boys exposed to chemotherapy by alkylating agents or hydroxyurea prior to TTC (36). Moreover, Medrano et al. reported a dose-dependent reduction in spermatogonia cells after exposure to alkylating agents, but also cytarabine and asparaginase (37). On the other hand, one study reported no significant difference in spermatogonia number between children previously exposed to gonadotoxic treatment and those who did not receive any previous therapy were observed in testicular samples using immunohistochemistry techniques (19). Recently, normal histology and presence of spermatogonia were observed in testicular tissue even after gonadotoxic therapy and just before conditioning for HSCT in patients, most of them diagnosed with high-risk or relapsed acute lymphoblastic leukemia (18). These results strengthen the concept that an opportunity for fertility preservation should also be offered to children with malignancy relapse or poor response to therapy. In our series the indication for fertility preservation was, in most of the cases, a disease relapse.

The reproductive safety of testicular tissue already exposed to chemotherapy needs to be addressed. Previous exposure to chemotherapy has not been shown to increase the risk of congenital birth defects in offspring of women after ovarian tissue auto-transplantation (38). Children born from childhood cancer survivors have not been found to have an increased rate of chromosomal abnormalities (39). Since no spermatozoa with reproductive potential have been developed from spermatogonial stem cells in humans, the reproductive safety of immature testicular tissue samples exposed to chemotherapy is lacking. Even if data on childhood cancer survivors are reassuring, more studies are needed to assess whether the use of immature testicular tissue cryopreserved after beginning chemotherapy is associated with an increased risk of congenital malformations and adverse neonatal outcomes.

The main limitation of our study is the short duration of follow up, which prevents us from drawing any conclusion on pubertal development and reproductive function after chemotherapy and TTC. Kanbar et al. have reported 139 testicular biopsies performed for fertility preservation between 2005 and 2020, including post-treatment FSH level for 57 patients and post-treatment semen analysis results for 27 of them (20). In those subgroups of patients, they observed higher than normal FSH level in 33% of the 57 patients and severely impaired semen parameters in 52% of the 27 patients. Pubertal onset (defined as a Tanner stage >1 and assessed at the time of the decision to perform the TTC) was an independent factor for testicular insufficiency. The same group has also reported that around 27% of children that complete testicular biopsy will be azoospermic after pubertal transition (26). It is unknown whether the reproductive impairment is merely due to the gonadotoxic therapy or, in part, also to the testicular tissue biopsy itself. In 112 males (median age of 8.6 years) who underwent orchiopexy and bilateral testicular biopsy for unilateral or bilateral undescended testis, reassuring data have shown that the biopsy was not associated with an increased risk of testicular microlithiasis, albuginea scars or testis masse and that no patient had developed antisperm antibody (mean age of 19.6 years) (40). Studies on the reproductive outcomes of children and adolescents treated with gonadotoxic therapies who did not undergo testicular tissue preservation can also help answer this question. In an unselected male population of long-term childhood cancer survivors (after high and low risk gonadotoxic chemotherapy), a high prevalence of oligospermia (20.6%) and azoospermia (17.7%) was observed with a higher prevalence in the high-risk subgroup (41). In a cohort of 55 boys having undergone unilateral testicular biopsy for fertility preservation, testicular volume and growth were similar compared to the contralateral testis at 1, 6 and 12 months of follow-up (42). Despite these reassuring data, more studies and longer follow-up are needed to better clarify issues such as reproductive safety of previously chemotherapy exposed immature testicular tissue and impact of testicular biopsy on pubertal development and reproductive outcomes.

Conclusion

The present data demonstrates that TTC in prepubertal and adolescent boys represents a safe procedure, with a low immediate complication rate, including in patients with disease relapse and poor response. In addition, the procedure was well-accepted among patients and families, with an acceptation rate of 90%. Although this procedure is experimental and future utilization currently remains hypothetical, TTC represents the only option to preserve future fertility in the pre-pubertal

male population and therefore should be offered to patients undergoing highly gonadotoxic treatment.

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 authors.

Ethics statement

This study was approved by the Local Ethics Committee (Commission Cantonale d'Ethique de Recherche sur l'être humain—CCER) (PB_2016-01378). Each center's protocol was also approved by their respective Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

CA, TD-F, NV, and FG-P conceived and designed the study and coordinated and supervised the data collection. DM, NV, and FG-P carried out the initial analyses. DM, AS, NV, and FG-P drafted the initial manuscript, reviewed, and revised the manuscript. CA, TD-F, CG, JB, IV, FB, KB, TB, M-PP, and MA critically reviewed the manuscript for important intellectual content and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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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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EDITED BY
Asma Chattha,
Mayo Clinic, United States

REVIEWED BY
Maria T. Bourlon,
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico
Peter Schlegel,
Weill Cornell Medical Center, NewYork-Presbyterian, United States
Romina Pesce,
Italian Hospital of Buenos Aires, Argentina

*CORRESPONDENCE
Mindy S. Christianson
mchris21@jhmi.edu

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Fertility preservation for pediatric patients with hemoglobinopathies: Multidisciplinary counseling needed to optimize outcomes

Bronwyn S. Bedrick[1], Taylor P. Kohn[2], Lydia H. Pecker[3] and Mindy S. Christianson[4]*

[1]Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2]Department of Urology, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [3]Department of Medicine, Division of Adult Hematology, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [4]Department of Gynecology and Obstetrics, Division of Reproductive Endocrinology and Infertility, Johns Hopkins University School of Medicine, Baltimore, MD, United States,

Hemoglobinopathies are autosomal recessive disorders that occur when genetic mutations negatively impact the function of hemoglobin. Common hemoglobinopathies that are clinically significant include sickle cell disease, alpha thalassemia, and beta thalassemia. Advancements in disease-modifying and curative treatments for the common hemoglobinopathies over the past thirty years have led to improvements in patient quality of life and longevity for those who are affected. However, the diseases, their treatments and cures pose infertility risks, making fertility preservation counseling and treatment an important part of the contemporary comprehensive patient care. Sickle cell disease negatively impacts both male and female infertility, primarily by testicular failure and decreased ovarian reserve, respectively. Fertility in both males and females with beta thalassemia major are negatively impacted by iron deposition due to chronic blood transfusions. Hematopoietic stem cell transplant (HSCT) is currently the only curative treatment for SCD and transfusion dependent beta thalassemia. Many of the conditioning regimens for HSCT contain chemotherapeutic agents with known gonadotoxicity and whole-body radiation. Although most clinical studies on toxicity and impact of HSCT on long-term health do not evaluate fertility, gonadal failure is common. Male fertility preservation modalities that exist prior to gonadotoxic treatment include sperm banking for pubertal males and testicular cryopreservation for pre-pubertal boys. For female patients, fertility preservation options include oocyte cryopreservation and ovarian tissue cryopreservation. Oocyte cryopreservation requires controlled ovarian hyperstimulation (COH) with ten to fourteen days of intensive monitoring and medication administration. This is feasible once the patient has undergone menarche. Follicular growth is monitored *via* transvaginal or transabdominal

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ultrasound, and hormone levels are monitored through frequent blood work. Oocytes are then harvested *via* a minimally invasive approach under anesthesia. Complications of COH are more common in patients with hemoglobinopathies. Ovarian hyperstimulation syndrome creates a greater risk to patients with underlying vascular, pulmonary, and renal injury, as they may be less able to tolerate fluids shifts. Thus, it is critical to monitor patients undergoing COH closely with close collaboration between the hematology team and the reproductive endocrinology team. Counseling patients and families about future fertility must take into consideration the patient's disease, treatment history, and planned treatment, acknowledging current knowledge gaps.

KEYWORDS

fertility preservation, sickle cell disease, beta thalassemia, oocyte cryopreservation, ovarian tissue cryopreservation, sperm cryopreservation

Introduction

Hemoglobin is an oxygen-carrying protein comprised of four subunits: two alpha chains and two non-alpha globin chains. In a healthy adult, approximately 95-98% of hemoglobin is in the form of HbA1, which consists of two alpha and two beta chains; the remaining small percentage of hemoglobin is in the form of HbA2 (two alpha and two delta chains) and HbF (two alpha and two gamma chains) (1). Almost 2,000 hemoglobin gene variants have been described (2). However, most variants are not associated with clinically significant disease. Indeed, it is estimated that 24% of the world population carry at least one altered globin gene, but only 5% carry a clinically significant variant (3). Hemoglobinopathies are autosomal recessive disorders: sickle cell disease, alpha thalassemia, and beta thalassemia.

Advancements in disease-modifying and curative treatments for the common hemoglobinopathies over the past thirty years have led to improvements in patient quality of life and longevity for those who are affected. However, the diseases, their treatments and cures may pose infertility risks. Expanding opportunities to preserve fertility in childhood are thus relevant for children with these common genetic conditions.

In this article, we discuss the indications and complications of fertility preservation in pediatric patients with hemoglobinopathies, specifically sickle cell disease and beta thalassemia. We also review their etiologies and impact on fertility and summarize their main disease modifying treatment options, focusing on the use of hydroxyurea and hematopoietic stem cell transplant. Finally, we review healthcare and research disparities in this field.

Overview of common hemoglobinopathies

Sickle cell disease

Sickle cell disease (SCD) refers to a group of hemoglobinopathies characterized by two β-globin gene mutations or deletions, at least one of which is the point mutation that leads to the production of hemoglobin S (HbS) (Table 1). An adenine-to-thymine substitution in the sixth codon of the beta-globin chain results in HbS. This substitution creates an insoluble polymer that distorts the cellular membrane and promotes the characteristic red blood cell sickling in deoxygenated states. The inability of HbS to deform normally results in hemolysis, a shortened red blood cell lifespan, and a hypercoagulable state. Additionally, the sickled red blood cells may become entrapped within vessels, leading to vascular occlusion and ischemia that promotes further sickling. This is the mechanism responsible for vaso-occlusive pain crises (VOC), acute chest syndrome (ACS), stroke, splenic sequestration, neuropathy, osteonecrosis, and recurrent infections, among other severe complications of SCD (4).

SCD occurs when an individual is homozygous for HbS (i.e., HbSS, sickle cell anemia) or compound heterozygous with another beta globin gene mutation.

Beta thalassemia

Thalassemia arises from globin chain imbalance due to mutations in one of the four alpha subunit genes or one of the two beta subunit genes. An imbalance in the production of alpha

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TABLE 1 Hemoglobinopathy pathophysiology, major treatment modalities, and impact on fertility.

Hemoglobinopathy	Pathophysiology	Fertility Effects	Treatments	Treatment Fertility Effect
Beta Thalassemia	Alpha globin chain precipitates due to globin chain imbalance, leading to hemolysis and ineffective hematopoiesis	Fertility effects thought to be secondary to chronic transfusions	Blood transfusions	- Iron overload leads to hypothalamic hypogonadism, impaired leptin synthesis, delayed puberty - Diminished sperm production
			HSCT	Chemotherapeutic agents and radiation may lead to diminished ovarian reserve, diminished sperm production, varying degree of gonadal failure and infertility
Sickle Cell Disease	Hemoglobin becomes insoluble polymer, distorts the cellular membrane and promotes red blood cell sickling in deoxygenated states; results in hemolysis, entrapment, and hypercoagulability	Male: - hypogonadism - impaired spermatogenesis - delay in sexual maturation - Erectile dysfunction Female - delay in sexual maturation - diminished ovarian reserve	Pain management: NSAIDs, opioids	Opioids: inhibits GnRH NSAIDs: impairs ovulation, fertilization, and implantation
			Blood/ exchange transfusions	- Iron overload leads to hypothalamic hypogonadism, delayed puberty
			Hydroxyurea	- Diminished sperm production and concentration - Diminished ovarian reserve - In pregnancy: birth defects, FGR
			HSCT	Chemotherapeutic agents and radiation may lead to diminished ovarian reserve, diminished sperm production, varying degree of gonadal failure and infertility

HSCT, hematopoietic stem cell transplant; GnRH, gonadotropin releasing hormone; NSAIDs, nonsteroidal anti-inflammatory drugs; FGR, fetal growth restriction.

and beta globin chains produces unpaired globin chains that precipitate within red blood cells, resulting in hemolysis and ineffective hematopoiesis. Thalassemia severity depends on the type of genetic defect (i.e., missense versus full deletion) and on the number of genes affected.

Beta thalassemia is caused by mutations in the beta globin gene. Some mutations reduce expression of the beta subunit (β+), whereas others result in complete loss of expression from that allele (β^0). Individuals with one functional beta globin (β/β+ or β/β^0; beta thalassemia minor) are asymptomatic carriers. Patients with some normal beta globin production (β+/β+ or $\beta+/\beta^0$; beta thalassemia intermedia) usually have mild to moderate anemia, although patients may require chronic transfusions. Beta thalassemia major (BTM) is characterized by severe anemia that results when both beta globin genes have deletions (β^0/β^0) or when a deletion is paired with another mutation that severely decreases beta globin expression (5).

When patients are dependent on transfusions for survival, regardless of genotype, they are said to have transfusion-dependent thalassemia (6). These patients usually have BTM and without treatment or cure, they are at risk of growth impairment, skeletal abnormalities, hepatosplenomegaly, and death within the first two decades of life (5). Life expectancy for individuals with BTM has significantly (7) improved over the years. In the 1970s, half of patients died before the age of 12 (8); however, many patients are now living into their 50s or 60s, making normal puberty important and parenthood viable (9).

Given the prevalence and severity of BTM and SCD, this review article will focus on these hemoglobinopathies. We note that a small, but growing number of people with alpha thalassemia major are surviving. These patients, like those with beta thalassemia major are at risk for iron overload and gonadotoxicity during HSCT. Given the lack of data, however, we do not focus on these patients (7, 10).

Impact of hemoglobinopathies on fertility

Sickle cell disease

Male infertility risks

Studies suggest that males with SCD are at risk for infertility as a result of both hypergonadatropic hypogonadism from vaso-occlusive induced testicular ischemia as well as hypogonadotropic hypogonadism from chronic transfusion-induced iron deposition in the hypothalamus and pituitary (11–13). Indeed, studies have demonstrated that approximately 24% of adult men with SCD are hypogonadal from both hypergonadatropic hypogonadism as well as hypogonadotropic hypogonadism (13–16). As a result, adolescent males may

experience a delay in sexual maturation by one to two years, and boys with more severe genotypes (HbSS, HbSβ^0) experiencing greater delays (13, 17).

Infertility in males with SCD can also occur as a result of severe erectile dysfunction. Vaso-occlusion of the corpus cavernosum can result in priapism and repeated vasoocclusive episodes with priapism can result in high rates of erectile dysfunction, with some studies demonstrating as many as 48% of men will have impaired erectile function at an average age of 28 years old (18). Priapism is a true SCD emergency as the risk of erectile dysfunction increases with prolonged episodes of priapism (19, 20). Severe cases of erectile dysfunction can make spontaneous reproduction difficult and limit future fertility and may even require penile prosthesis to achieve an erection necessary for intercourse.

Even in eugonadal men with SCD, spermatogenesis is often affected; impaired semen parameters have been observed in men with normal FSH, LH and testosterone levels, possibly due to testicular infarction (21, 22). In one study, 91% of patients with SCD and taking no disease modifying therapy had an abnormality on semen analysis. The most common abnormality being impaired motility, though total motile counts were still on average around 32 million motile sperm (23). Despite a high published rates of abnormalities on semen analysis in men with sickle cell disease, in a large retrospective registry study of patients with sickle cell disease, Gordeuk et al. found that among 1018 men with sickle cell disease, 620 pregnancies conceptions had been reported for a rate of 0.61 per man (24).

Men with SCD can conceive an unassisted pregnancy with a partner, though no largescale study has assessed the frequency of infertility in this population, and further studies are needed to determine.

Female infertility risks

The majority of data on female sexual development in SCD is from the 1960s-1990s. These studies demonstrated that females with SCD achieve sexual development and undergo menarche at later ages than unaffected females (25), with more severe genotypes (HbSS, HbSβ^0) having greater delays than those with less severe hemoglobinopathies (HbSβ^+ or HbSC) (13, 26). The delay in menarche is thought to be constitutional (13, 26), and age of menarche is consistent with bone age (27). Once menarche is reached, however, patients can be expected to have regular menstruation (26). The effects of disease-modifying therapy on age of menarche remains poorly defined (28).

The extent to which fertility is impacted in female patients with SCD is also unclear. Historically, lower pregnancy rates among women with SCD was used as a surrogate for fertility (29), but this approach to estimating fertility is limited. Women with SCD have multiple risks for reduced ovarian reserve: chronic inflammation, oxidative stress, and ovarian ischemia and reperfusion injuries (30, 31). Three studies have demonstrated normal anti-müllerian hormone (AMH) levels in untreated adolescent and young adults with SCD (32–34). However, women with SCD experience a more rapid decline in ovarian reserve, with lower levels of AMH than age matched controls (33, 35, 36). Females with SCD develop diminished ovarian reserve (DOR) at younger ages (25-30 years) than age-matched women (33, 34). Yet, no studies to date define the definitive risk of infertility in this population (28). Interestingly, in 2021 Mamsen et al. evaluated ovarian health markers in adolescent females with hemoglobinopathies. They found no difference in ovarian follicular density, morphology, and expression of follicular and oocyte proteins between those with SCD and health age-matched controls, suggesting that the primordial follicle pool is normal in this population (37).

Beta thalassemia

Delayed sexual development, menarche, and hypothalamic hypogonadism are common in adolescents with BTM (38) but are these thought to be secondary to iron deposition from chronic transfusions rather than a consequence of the disease itself (39). Impact of chronic transfusion on pubertal development and fertility and prevention options will be further discussed in the next section.

For both SCD and BTM, a patient's disease and disease severity, may impact which fertility preservation treatment options are available to them and the success of their fertility treatment. When counseling patients and guardians about treatment options, it is important to consider how the individual's unique disease presentation may impact success.

Palliative and disease modifying therapies as potential infertility risks

Pain management

Chronic pain and opioid use are common sequelae of SCD. Additionally, almost 70% of patients with BTM report recent pain (40). Opioids have been shown to suppress the hypothalamic-pituitary-gonadal axis through inhibiting gonadotropin releasing hormone (41). Indeed, women taking opioids chronically have an approximately 50% reduction in estradiol and testosterone levels, a 30% reduction in gonadotropins (42), and may experience menstrual cycle disruption (42). Chronic opioid use also reduces testosterone levels in males (43) and leads to lower sperm motility and morphology (44) as well as increased DNA fragmentation (43).

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) can impact fertility through inhibition of cyclooxygenase 2 (COX-2), leading to reduced prostaglandin synthesis and

impairments in ovulation, fertilization, and implantation (45, 46). While small studies have linked impaired ovulation and infertility with NSAID use, the impact of chronic NSAID use on ovarian reserve and fertility is not well understood (30). Furthermore, polypharmacy may obfuscate associations between analgesic medications and fertility in patients with SCD or BTM.

Blood transfusion

Red blood cell transfusion or exchange is a cornerstone for symptomatic management and prevention in patients with SCD and BTM. For patients with SCD, these transfusions/exchanges dilute sickle cell hemoglobin and thus reduce the sequalae of sickling. For patients with BTM, transfusions supply normal red blood cells and inhibit ineffective erythropoiesis (47). These patients often undergo transfusions every three to four weeks (5).

Despite clear benefits, chronic transfusions often lead to iron overload, which has both direct and indirect impact on gonadal function. Interestingly, iron deposition appears to be a greater issue for individuals with BTM than those with SCD (48, 49), and effects from iron deposition are the major complications associated with BTM. For example, in a study of 73 patients with BTM and SCD who received chronic transfusions, 33% of BTM had gonadal failure compared to 0% of SCD patients (48). A similar 2006 study found that 40% of patients with BTM had hypogonadism, and they were eight times as likely to have hypogonadism as patients with SCD (49). Chelation therapy thus becomes essential for those receiving chronic blood transfusions and should often be initiated prior to puberty in order to encourage normal development (50). However, progressive deposition in the hypothalamus and pituitary will occur even in the setting of chelation therapy (51).

Hypothalamic hypogonadism may result from direct iron deposition in the hypothalamus, pituitary, and reproductive organs as well as free radial oxidative stress (39, 52–54). The anterior pituitary is particularly sensitive to iron deposition and demonstrates evidence of iron accumulation within the first decade of life. Damage to the anterior pituitary leads to disturbances in gonadotropic hormones and may lead to pubertal delays or arrest. In fact, hypothalamic hypogonadism is the most common endocrinopathy affecting individuals with BTM (38). It is estimated that 70% of boys with beta thalassemia intermedia or major will develop hypogonadotropic hypogonadism (55), and over 50% of females will not reach menarche spontaneously (38). In addition to iron deposition in the hypothalamus and pituitary, gonadal iron deposition may occur (56). However, ovarian function appears to be preserved as evidenced by an age-appropriate ovarian response to hyperstimulation (57, 58). Furthermore, ovarian tissue preserved for fertility preservation in females with BTM demonstrate normal follicular density and morphology (37).

Iron deposition also negatively impacts fertility in patients with BTM include impaired leptin synthesis and disruption of liver and pancreatic function, which are involved in hormone and antioxidant metabolism (39). It has been suggested that iron deposition in adipose tissue disrupts the production of leptin, a hormone now believed to be vital for the pubertal development. In a study of 101 adolescents with BTM, Perrone et al. found significantly lower leptin levels than expected for Tanner stage 1-4 males and stage 3-5 females (59). In a separate study, Dedoussis et al. found that leptin serum levels were significantly lower for BTM patients who received either sporadic or chronic transfusions than normal and that leptin level was negatively correlated with levels of transferrin reception for those who were transfusion dependent (60).

Males with transfusion dependent beta-thalassemia have high rates of oligospermia and azoospermia, but conception is still possible. In a study of 52 men, 60% were normospermic, 17% were oligospermic, and 23% were azoospermic (61). For men with impaired spermatogenesis, spermatogenesis can be induced with exogenous gonadotropin stimulation, with human chorionic gonadotropin (hCG) alone or combined with human menopausal gonadotropin, thus making paternity possible (9, 62–64) Indeed, in a survey of ten thalassemia centers, including 738 transfusion-dependent men over the age of 18, 75% of those married or living with a partner conceived a pregnancy within the first two years of the marriage. Of these pregnancies, 79% occurred *via* natural conception and 15% of men required exogenous gonadotropin stimulation (65).

Hydroxyurea

Approved by the FDA in 1998 for use in adults with sickle cell anemia, hydroxyurea has dramatically improved patient quality of life and reduced disease complications. Patients taking hydroxyurea are less likely to be hospitalized or require transfusions. Studies have also found improvements in long-term survival and reduced risk of stroke (66–71).

Hydroxyurea inhibits ribonucleotide reductase and thus cell cycle specific DNA replication. Through unclear mechanisms, hydroxyurea shifts expression of the beta globin locus resulting in increased production of HbF and decreased production of HbS. As a result of decreased HbS concentration, hemoglobin is less prone to polymerization and sickling. Hydroxyurea also decreases circulating leukocytes and reticulocytes, increases red blood cell volume, and improves cellular deformability, thereby reducing painful events (72).

Given substantive improvements in patient symptoms and markers of disease control, the National Heart, Lung, and Blood Institute (NHLBI) recommends offering hydroxyurea in pediatric patients over 9 months of age, regardless of clinical severity (68). However, the optimal time to start hydroxyurea therapy has not been established, and other national guidelines recommend starting at later ages (73). Additionally, in patients

not taking hydroxyurea, it may be recommended prior to bone marrow transplant to reduce the risk of rejection and improve chance of engraftment (74). While there have been some studies suggesting benefit in the use of hydroxyurea in patients with BTM (75), these results are not widespread and its use in this population is uncommon (5).

Hydroxyurea use may be lifelong for patients with SCD. While there is strong evidence as to the myelosuppressive effects of hydroxyurea, data on other long-term effects, such as on infertility, are conflicting. The National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR) gave hydroxyurea use in pregnancy its second highest concern level due to risk for birth defects and intrauterine growth restriction (76). CERHR also ranks use of hydroxyurea in post-pubertal males as "highly concerning."

For men, hydroxyurea owes its rank of "highly concerning" to its impact on sperm parameters. In men, treatment with hydroxyurea has been shown to significantly impair total sperm concentration (pretreatment: 38.5 million sperm/mL; post treatment: 18.46 million sperm/mL) but forward motility remains similar (pretreatment: 28.6%; post treatment: 29.4%) (23). In another study of men with SCD on hydroxyurea therapy, 20% developed oligospermia and 10% developed azoospermia (77). The impact on spermatogenesis extends to prepubertal males as well. In one study, two young males initiated on hydroxyurea at ages 10 and 16 were found to have severe oligospermia eight years after treatment initiation; two others who began hydroxyurea at ages 8 and 11 were found to be azoospermic 15 and 12 years later, respectively (78).

Despite hydroxyurea's deleterious impact on semen parameters, studies have shown normalization of semen parameters after discontinuing the medication. In one study, almost 75% of men who stopped hydroxyurea for three months had normalization of their semen parameters (77). In a recent study by Joseph et al., there was no difference in semen volume, sperm concentration, total sperm count, or spermatozoa motility, morphology, and vitality between men who received hydroxyurea prior to puberty and men who were hydroxyurea naïve. In this study, men who had a history of hydroxyurea use stopped hydroxyurea on average two and a half years prior to semen analysis (79). These studies suggest that while hydroxyurea may have more severe effects when started in the prepubertal period, the effects are potentially reversible. However, the reliability and duration to recovery of spermatogenesis after hydroxyurea has not been well elucidated, and so sperm banking or testicular tissue banking may be considered prior to initiating hydroxyurea therapy.

While hydroxyurea is considered 'low risk' for infertility in women, it is associated with diminished ovarian reserve in three small studies of people with sickle cell anemia (hemoglobin SS and hemoglobin S beta-null thalassemia). During the decade-long follow up of the Multicenter Study of Hydroxyurea (MSH) randomized control trial, AMH was lower for women who were on hydroxyurea than for those not taking it (33). In 2015, Elchuri et al. compared 10-21 year-old females with SCD who received supportive care, hydroxyurea, or underwent bone marrow transplantation. They found that 24% of patients treated with hydroxyurea had diminished ovarian reserve as defined by AMH <5% of expected for age-matched controls (32). Moreover, among patients taking hydroxyurea, those with DOR had been taking the medication for 2.8 years longer on average than those without DOR. No patient receiving hydroxyurea in this study met criteria for premature ovarian insufficiency as defined as FSH is > 40 IU/L (32). In another single center analysis of ovarian reserve in 26 women with sickle cell anemia, all (n=5) women diagnosed with DOR were taking hydroxyurea (34). Whether markers of ovarian reserve normalize when hydroxyurea is stopped and whether hydroxyurea impairs fertility, ovarian hyperstimulation, or oocyte quality is not established. Given the potential to affect oocyte quantity and quality, the potential risks associated with its use must be weighed against the potential benefit of reduced rejection and graft versus host disease risk.

Hematopoietic stem cell transplant

Hematopoietic stem cell transplant (HSCT) is currently the only curative treatment for SCD and transfusion dependent beta thalassemia. It is recommended when symptoms are no longer controlled with supportive care and medical management or for those who with serious disease complications. For patients with SCD who underwent HLA-matched transplants, greater than 90% sustained engraftment and resolution of painful crises (80). Similar improvements are seen in patients with BTM. To date, more than 5,000 of HSCT have been performed for these disorders (81–83).

For patients with SCD, HLA matched sibling donors result in the highest event-free survival. However due to limited availability of HLA-matched siblings, haploidentical, matched unrelated, and mismatched unrelated donation are possible and mostly offered on an experimental basis (84). Age at transplantation impacts morbidity and mortality, which is an important consideration for fertility preservation. When HSCT is performed prior to 16 years of age, the 5-year survival is 95% compared to 81% at older ages (82), and children <10 years old have decreased mortality compared to those transplanted 10-21 years old (85). Similarly, for BTM HLA identical transplantation at younger ages (<14 years) improves outcomes and survival (83, 86) due to higher rates of disease complications with age; transplant related mortality is <5% if performed prior to 5 years old (83).

A variety of condition regimens for HSCT are described. The first successful regimens were myeloablative. However, given the intolerability of these regimens for those with severe disease, nonmyeloablative conditioning regimens have been developed. Many of the conditioning regimens contain chemotherapeutic agents with known gonadotoxicity, such as cyclophosphamide. Whole body radiation may also be included. The commonly

used radiation dose (2-3Gy) is less than the effective sterilizing dose (>14 Gy in females; > 6 Gy in males) but falls within ranges known to cause significant gonadal damage (87–89). Despite the potential for significant deleterious gonadal damage, most clinical studies on toxicity and impact of HSCT on long-term health do not evaluate gonadal function or fertility. From the studies that do (Table 2), it is evident that gonadal failure is common, but the impact is variable and unpredictable (115). Additionally, younger age at transplant and male sex appear to reduces risk of gonadal failure (109).

After transplantation, patients are often placed on additional immunosuppressive medications to reduce the risk for GvHD, such as cyclophosphamide or sirolimus (84, 116). Primary ovarian insufficiency is a common consequence of cyclophosphamide (117, 118), although ovarian reserve appears to be less affected if cyclophosphamide is given prior to menarche (119). Sirolimus has also been shown to significantly reduce sperm counts and fertility rates in male organ transplant recipients (120), lead to gonadal dysfunction and secondary infertility (121), and to negatively impact IVF outcomes (122).

In summary, counseling patients and families on future fertility is complicated by the wide range of conditioning regimens available and poor reporting on gonadal function post-transplant. Furthermore, impact on gonadal function may be highly variable even for the same conditioning regimen, with some patients experiencing rapid gonadal failure necessitating hormonal supplementation and others retaining full function. Long term follow-up and a greater understanding of the interplay between gonadotoxic treatment and gonadal function is needed.

Fertility preservation

Given the risk of gonadal failure after HSCT, fertility preservation before HSCT should be offered (123). Early counseling, no matter the patient age, is vital to provide patients with the optimal opportunity to protect their reproductive potential. Furthermore, fear of toxicity and sterility are important barriers to HSCT acceptance (124, 125). However, significant access to care remains including provider awareness, patient/family preferences, and financial barriers.

Male fertility preservation

Fertility preservation is an important consideration for men and their provider to discuss prior to HSCT or even initiating hydroxyurea therapy (126). Male fertility preservation in many ways is more straight forward than for females - ejaculate or testicular tissue do not require stimulation cycles and can be collected nearly immediately, but counseling men about the risk of gonadotoxic agents is less frequently discussed with males than female cancer patients (127, 128).

Sperm banking

For post pubertal males, sperm banking can readily be performed *via* ejaculation which allows sperm to be frozen until the patient desires fertility, at which time *in vitro* fertilization could be pursued (129). Given the impact of hydroxyurea on spermatogenesis, it is recommended that hydroxyurea be discontinued several months prior to sperm collection (123). This may allow for normalization of sperm parameters. However, discontinuing hydroxyurea for several months may be challenging for patients with severe SCD. While the process of sperm cryopreservation is relatively simple, informing patients of the risk of gonadotoxic agents is not always discussed or pursued (130). Studies have found low rates of fertility preservation counseling prior to gonadotoxic chemotherapy for cancer (128). However, these findings may not be applicable to adolescent males facing gonadotoxic treatment for their chronic lifelong disease and more research is needed about fertility preservation counseling in this population.

Discussion of fertility preservation and increasing state-mandated coverage of fertility preservation has resulted in an increase in rates of fertility preservation (128). In recent years, several online based companies have emerged that allow for in-home collection for sperm banking wherein the individual receives a kit, ejaculates in their own home into the provided container, and then ships the kit back for cryopreservation (131). With increasing insurance coverage of fertility preservation, awareness of fertility preservation options amongst providers, and online-based sperm preservation companies, hopefully the rates of sperm banking prior to gonadotoxic agents will increase.

Testicular tissue preservation

Fertility preservation is more complex if the male has not yet gone through puberty. Indeed, rates of azoospermia are quite high in boys 13 years or younger (132). In these cases, surgery is required to harvest testicular tissue for cryopreservation (133). Only a small amount of testicular tissue is collected, and the procedure is well tolerated with minimal side effects (134). It is important however to note that there is no current ability to use this sperm for future fertility attempts; thus prepubescent testicular preservation is only offered in certain academic centers as part of a research protocol (135). Research into maturation of the testicular tissue and methods of reimplantation are ongoing. The field recently took a major step forward with the successful transplant of frozen rat spermatogonial stem cells into recipient mice, which produced differentiating germ cell types with production of spermatids (136). While this murine study is certainly promising for the future of reimplantation of testicular tissue, parents of boys undergoing gonadotoxic treatments should be aware that much research is still required and previously harvested testicular tissue may not be ready for spermatogenesis at the time when fertility may be desired. Currently, testicular tissue preservation

TABLE 2 Clinical studies of hematopoietic stem cell transplant in patients with sickle cell disease and beta thalassemia that evaluated reproductive function.

Authors, year	Diagnosis	Sample Size	Median age at time of HSCT (range)	Conditioning regimen	Follow up time	Impact on reproductive function
De Santis et al., 1991 (90)	BTM	N=30	Mean 12.9 years (9.3-17.2 years)	Busulfan/cyclophosphamide	0.7-5.1 years	- 12/15 females had elevated levels of LH and FSH - 15/15 males had normal LH, FSH, testosterone
Vermylen et al., 1998 (91)	SCD	N=50	7.5 years (0.9-23 years)	Busulfan/Cyclophosphamide +/- total lymphoid irradiation or ATG	0.3-11 years	- 2/2 postpubertal females developed secondary amenorrhea - 5/6 prepubertal females had primary amenorrhea and required hormone replacement - 1/6 prepubertal female had spontaneous menarche - 6/6 males adolescent boys had normal sexual development - 4/6 males had decreased testosterone and elevated FSH -1/6 males had elevated LH
Slavin et al., 1998 (92)	Malignant and non-malignant hematologic diseases	N=26 (1 with BTM)	31 years (1-61 years)	Fludarabine/ATG/low-dose busulfan	Median: 8 months	1 19-year-old female regained menstruation
Bernaudin et al., 2007 (93)	SCD	N=87	9.5 years (2-22 years)	Busulfan/cyclophosphamide/ ATG	2-17.9 years	- 7 postpubertal females developed amenorrhea, low estradiol, elevated FHS and LH levels - "most" of prepubertal females required hormone therapy - 2 prepubertal girls underwent spontaneous puberty - All males had normal testosterone, FSH, and LH levels
Brachet et al., 2007 (94)	SCD	N=30	7.2 years (2.3-14.2 years)	Busulfan/cyclophosphamide	2.5-17.3 years	- 7/10 females had amenorrhea - 1 spontaneous pregnancy/live birth - 9/9 males underwent spontaneous puberty 9/9 males had normal/low-normal testosterone -1/2 males had azoospermia
Lukusa et al., 2009 (95)	SCD	N=10	32 years (10-34 years)	Busulfan/cyclophosphamide +/- total lymphoid irradiation	8-21 years	- 5/5 spontaneous puberty - 3/6 azoospermia - no pregnancies fathered
Hsieh et al., 2009 (96) (supplemental material)	SCD	N=10	26 years (16-45 years)	Total-body irradiation (3Gy)/alemtuzumab	15-54 mo	1.25 to 4.5 years post HSCT: - Range of FSH 5.8-179 units/L; LH 2-98.4 units/L - 1 female patient had FSH >40 units/L 0.5 years after HSCT; < 40 m/L FSH 1 and 2 years after - 1 pregnancy/ delivery - 1 female has regular menses on oral contraception - Range of total testosterone 191-1230 ng/dL; free testosterone 4.1-40.7 ng/dL

(Continued)

TABLE 2 Continued

Authors, year	Diagnosis	Sample Size	Median age at time of HSCT (range)	Conditioning regimen	Follow up time	Impact on reproductive function
						- 1 male on testosterone replacement
Walters et al., 2010 (97)	SCD	N=55	<16	Busulfan/cyclophosphamide	3-12.4 years	- 9/12 females had amenorrhea - 2 spontaneous pregnancies/ live births - 8/11 males had low testosterone
Majumdar et al., 2010 (98)	SCD	N=10	10.1 years (2.8-16.3 years)	Busulfan/cyclophosphamide/ thyroglobulin	2.9-11 years	-2/7 had FSH >40 mIU/mL more than 3 years post-transplant -2/3 females >14 have POF
Dallas et al., 2013 (99)	SCD	N=22	11.1 years (5.4-17.4 years)	Busulfan/cyclophosphamide/ thyroglobulin	0.9-12.3 years	-5/9 males had normal gonadal function (normal LH, FSH, testosterone) - 3/9 males had hypogonadism with normal testosterone -1/9 male required testosterone - 2/4 females developed POF requiring therapy -2/4 females had normal cycles and hormone levels - 1 pregnancy (unspecified if spontaneous)
Hsieh et al., 2014 (100)	SCD	N=30	Not reported (16-65 years)	Alemtuzumab/total-body irradiation (300 cGy)/ sirolimus	1-8.6 years	- spontaneous conception for 2 women and 2 men after transplant (no specifics)
Bhatia et al., 2014 (101)	SCD	N=17	8.9 years (2.3-20.2 years)	Busulfan/fludarabine/ alemtuzumab	135–2731 days	- Semen analyses, testosterone, LH, FSH levels measured in postpubteral males - AMH, estradiol, LH, FSH levels measured in postpubertal females No results reported
Dedeken et al., 2014 (102)	SCD	N=50	8.3 years (1.7-15.3 years)	Busulfan/cyclophosphamide; Busulfan/cyclophosphamide/ ATG	0.4-21.3 years	- 3/12 spontaneous puberty - 1 female had 2 spontaneous pregnancies - 1 female had POF which recovered spontaneously and had normal pregnancy
Soni et al., 2014 (103)	SCD	N=15	5 years (1.5-18 years)	Busulfan/cyclophosphamide/ thyroglobulin	0.9-7.5 years	-2/3 females had gonadal dysfunction
Maheshwari et al., 2014 (104)	SCD	N=16	6.2 years (1.2-19.3 years)	Busulfan/cyclophosphamide/ thyroglobulin	1.3-9 years	-2/5 (1 male and 1 female) had gonadal dysfunction requiring hormone replacement
Elchuri et al., 2015 (32)	SCD	N=56	10-21 years	Supportive care vs hydroxyurea (≥20mg/kg for ≥12mo vs HSCT (busulfan and cyclophosphamide)	n/a	- Mean AMH was 17.1 (supportive care), 13.4 (HU), and <0.57pmol/L (HSCT) - POI was found in 0% (Supportive care and HU) and 89% (HSCT) patients
King et al., 2015 (105)	SCD, BT	N=52	11.5 years (0.8-20.3 years)	Reduced intensity conditioning: alemtuzumab/ Fludarabine/melphalan	0.75-11.8 years	- Resumption of menses within a year of transplant in 4 teenagers - "maintained fertility" (not defined) in 3 females

(Continued)

TABLE 2 Continued

Authors, year	Diagnosis	Sample Size	Median age at time of HSCT (range)	Conditioning regimen	Follow up time	Impact on reproductive function
Madden et al., 2016 (106)	Mixture of diagnoses (immune, metabolic, hemoglobinopathy) Results not differentiated by diagnosis	N= 43 (10 had hemo-globinopathy)	3.4 years (1.5 mo-20 years)	Reduced intensity conditioning with Alemtuzumab/ Fludarabine/mephalan	2 to 8 years	- 1 of 17 had hypogonadism (also had chronic GvHD) - 3 of 3 postpubertal girls resumed menstruation; 2 had normal pregnancies - 9 of 11 age-appropriate Tanner development
Marzollo et al., 2017 (107)	SCD	N=11	6.5 years (4-16.3 years)	Treosulfan/fludarabine/ATG/ thiotepa	0.8-6.5years	- 3/4 had normal pubertal development -1/4 had secondary hypogonadism
Santarone et al., 2017 (108)	BTM	Males: N=8 Females: N=15	Males: 15 years (4-24 years) Females: 14 years (2-21 years)	Busulfan (total dose, 14 mg/kg) and cyclophosphamide (total dose, 200 mg/kg) as conditioning therapy and cyclosporine and short-course methotrexate as GvHD prophylaxis	Median 24 years (10-33 years)	-15 women achieved 27 pregnancies, 21 were achieved *via* natural conception, 6 *via* IVF - 2 miscarriage - 3 abortions (2 intended, 1 unintentional) - 22 live births - 8 men achieved 15 pregnancies with their partner, all *via* natural conception - 1 intended abortion - 14 live births
Rahal et al., 2018 (109)	BT	N=99	5.9 years (8mo-26 years)	Busulfan/cyclophosphamide; busulfan/fludarabine +/- thiotepa 3 other regimens including radiation	2-30 years	- Hypogonadism present in 56% of females and 14% of males - 6/6 females had secondary amenorrhea; 5 had hypogonadism -12/33 females had spontaneous and normal puberty -21/33 females had delayed puberty - 11/27 females had 1+ successful pregnancy, 2 required oocyte donation (both had delayed puberty) -4/22 males had delayed puberty; 3 developed hypogonadism - 18/22 males had normal pubertal development -4/21 males had fathered 1+ children (1 required IVF for hypogonadism and oligo-asthenozoospermia)
Zhao et al., 2019 (110)	SCD	N=3	14 years (11-15 years)	Alemtuzumab/fludarabine/ melphalan	>1 year post transplant	-3/3 normal testosterone - 2/3 azoospermia - no pregnancies fathered
Elchuri et al., 2020 (111)	SCD	N=40	9 years (6-34 years)	Busulfan/cyclophosphamide	1.1-18.5 years	- 21/21 females had DOR; 18 had undetectable AMH - 10/21 females had POI - 1 female had a spontaneous pregnancy/livebirth - 16/16 males had normal

(Continued)

TABLE 2 Continued

Authors, year	Diagnosis	Sample Size	Median age at time of HSCT (range)	Conditioning regimen	Follow up time	Impact on reproductive function
						testosterone - no males fathered pregnancies
Bernaudin et al., 2020 (112)	SCD	N= 234	8.4 years (2.2-28.9 years)	Busulfan, cyclophosphamide at 200 mg/kg and rabbit ATG at different doses	0.1-27.6 years	- 14/14 postpubertal females were amenorrheic within 1 year and required hormone replacement - "Most" of 32 pre-pubertal females required hormone therapy - 9 of 32 prepubertal girls underwent spontaneous puberty - 6 spontaneous pregnancies - 2 females had orthotopic ovarian fragment autograft; both recovered ovarian function; 1 conceived twice - All males who were of pubertal age had normal development, normal testosterone, FSH, LH - 3 males had fathered spontaneously
Rostami et al., 2020 (61)	BTM	N=43 (HSCT) N=52 (chronic transfusion)	Range 16-41 years	Cyclophosphamide/busulfan		- 33% of entire cohort had hypogonadism HSCT cohort -26% had dry ejaculate - 51% had azoospermia - 12% had oligospermia Transfusion cohort - 10% had dry ejaculate - 23% had azoospermia -17% had oligospermia
Alzahrani et al., 2021 (113)	SCD	N=122	29 years (10-65 years)	Alemtuzumab/total body irradiation (3 Gy)	0.6-14.9 years	- 7 females had 1+ spontaneous pregnancies - 7 males fathered 1+ pregnancies spontaneously
Boga et al., 2022 (114)	SCD	N= 49	Not reported (18-45 years)	Busulfan/cyclophosphamide/ Fludarabine/total body irradiation (2Gy)	>2 years after transplant	- 15/22 females had documented amenorrhea - 7/22 females without amenorrhea were on hormonal support - All women had AMH <1ng/mL 2 years after transplant - 10/22 females had FSH >40 IU/mL x2 - 1 female had spontaneous pregnancy and miscarriage - 1 pregnancy from embryo cryopreservation -74% of males had azoospermia - testosterone levels were all normal - 4/21 males fathered pregnancies; 1 required IVF

ATG, anti-thymocyte globulin; AMH: anti-mullerian hormone; FSH, follicle stimulating hormone; GvHD, graft vs host disease; HSCT, hematopoietic stem cell transplant; HU, hydroxyurea; IVF, in vitro fertilization; LH, luteinizing hormone; POF, primary ovarian failure; POI, primary ovarian insufficiency.

is recommended for patients at significant risk for infertility, including patients with SCD and BTM (137). Operative considerations are discussed below.

Female fertility preservation

The most important factors determining mode of fertility preservation in a female patient are whether she has undergone menarche and the urgency with which the gonadotoxic treatment is needed. Fertility preservation prior to HSCT for hemoglobinopathies is not usually urgent. This will allow for improved coordination and health optimization prior. In some cases, this may allow time for menarche to occur and thus permit the use of controlled ovarian hyperstimulation and oocyte cryopreservation. For patients who have not yet undergone menarche and for whom waiting until after menarche for transplantation is not feasible due to patient age and disease severity, ovarian tissue cryopreservation (OTC) is the only current option for fertility preservation. For females who have undergone menarche, OTC and oocyte cryopreservation are available. While embryo cryopreservation is another option, it is less likely in the pediatric and young adult population as it requires a sperm source and has greater ethical implications.

Oocyte cryopreservation

Controlled ovarian hyperstimulation (COH) requires ten to fourteen days of intensive monitoring and medication administration. Follicular growth is monitored *via* transvaginal or transabdominal ultrasound, and hormone levels are monitored through frequent blood work. Oocytes are then harvested *via* a minimally invasive approach under anesthesia.

Common complications of COH include headache, nausea, abdominal distention, and discomfort. Less commonly, ovarian hyperstimulation syndrome (OHSS) occurs, which may produce venous thromboembolism (VTE), ascites, and cardiopulmonary effusions (138). These consequences of COH are of even greater concern for patients with hemoglobinopathies, who may have altered pain perception and be less able to tolerate the discomfort of COH. Ovarian hyperstimulation syndrome also creates a greater risk to patients with underlying vascular, pulmonary, and renal injury, as they may be less able to tolerate fluids shifts (139). Indeed, COH increases the risk of VOC and ACS in patients with SCD. To date, there are 4 reported cases of acute pain crises during COH (Table 3).

Results of COH in patients with SCD are variable, with the number of oocytes retrieved ranging from 4 - 31. Fifteen is considered the minimum number of oocytes to harvest to optimize the change of pregnancy in one cycle (152). However, only 25% of the reported patients reached this goal. No patients in these cohorts underwent multiple cycles for fertility preservation, perhaps reflecting time and monetary constraints (139). There is also a scarcity of published data on ovarian stimulation protocols and outcomes in adolescent patients with and without hemoglobinopathies (153). It is generally recommended to use adult dosing regimens as a guide, adjusting for age, FSH level, and AFC. However, this may require frequent and significant dose adjustments. For example, in their cohort of eight teenage girls, Lavery et al., reported that dose adjustments were needed in 80% of cases (154).

To date, there have been several reports of successful and uncomplicated ovulation induction and IVF cycles for untransplanted patients with BTM. However, there have been no published COH protocols for BTM prior to fertility preservation (155, 156). These authors recommended discontinuing iron chelators prior to ovulation induction (156) as they are contraindicated in pregnancy, but this is not necessary for the purpose of fertility preservation.

Ovarian tissue cryopreservation

Ovarian tissue cryopreservation (OTC) is an increasingly utilized method of FPT. As of 2019, OTC is no longer considered experimental by the American Society for Reproductive Medicine (ASRM), although it may be in other countries. As of 2017, there had been 130 live births from OTC (157), with estimates of greater than 200 births as of 2020 (158). Given the younger ages at which HSCT is recommended for patients with hemoglobinopathies, OTC may be the only mode of FPT available.

Ovarian tissue cryopreservation is commonly performed *via* an outpatient laparoscopic surgery in which an ovary, or portion of the ovary is removed. This tissue is then stored until future use at which point ovarian tissue transplantation (OTT) may occur. Since OTC enables preservation of a larger cohort of primordial follicles, ovarian endocrine function may be restored after OTT. Indeed, in a metanalysis of 309 cases of OTT, endocrine restoration, as defined by cyclic menstrual cycles, ovarian follicle growth on ultrasound, or pregnancy, was achieved in 64% of cases. Clinical pregnancy rate after OTT was 57.5% (159).

Importantly, ovarian endocrine function appears to be restored in the small number of reported post-OTT patients with hemoglobinopathies (Table 4). In 2006, Donnez et al. was the first to report restoration of ovarian function after orthotopic transplantation in a patient with HbSS. The patient underwent OTC prior to HSCT at age 21 years old. She required hormone supplementation after transplant and eventually had an OTT after which she had resumption of ovarian function, evidenced of follicular development, and regular menstruation (140). These findings have been replicated in several other reports of adolescent patients with hemoglobinopathies, including patients who were prepubertal (37).

TABLE 3 Clinical reports of ovarian tissue cryopreservation for sickle cell disease and beta thalassemia.

Author, Year	Cases	Age	Diagnosis/ Genotype[1]	Indication for FPT	OTC	Post HSCT	Need for hormonal supplementation	Outcomes
Donnez et al., 2006 (140)	1	21 years	HbSS	Prior to HSCT (busulfan/ cyclophosphamide)	LSCO	Amenorrheic; FSH 48.2 mIU/mL; LH 18.5 mIU/ml; Estradil <10 pg/ml	Required	- After cessation of HRT, bimonthly FSH, LH, 17beta-estradiol demonstrated anovulation -Part of cryopreserved ovary reimplanted - Resumption of ovarian function, follicular development, menstruation
Roux et al., 2010 (141)	1	20 years	HbSS	Prior to HSCT (busulfan/ cyclophosphamide)	LSCO	Clinical and biological POI	Required	Desired pregnancy; had transplant - 4 months after OTT, follicle development - 19 weeks after OTT, stopped HRT and normalized AMH, FSH -spontaneous pregnancy, uncomplicated
Revel et al., 2011 (142)	1	19 years	BTM	Prior to HSCT	LSCO	Clinical and biological POI	Required	- Desired pregnancy; 5 mature oocytes were thawed but did not mature - After 1st OTT, FSH decreased, estradiol increased - After 3 cycles of failed IVF, ovarian tissue stopped responding to induction - Underwent 2 additional OTT - after 14th cycle of IVF, conceived, delivered full term
Revelli et al., 2013 (143)	1	21 years	Transfusion-dependent BT	Prior HSCT (busulfan/ cyclophosphamide)	Ovarian cortex harvest	Clinical and biological POI	Required	- Desired pregnancy; discontinued HRT, (FSH 72.3 IU/L, LH 32.1 IU/L) with a drop of E2 levels (12 pg/mL) - 3 months after OTT, E2 79 pg/mL, and FSH levels decreased 46.1 IU/L - Spontaneous pregnancy, full term cesarean delivery
Demeestere et al., 2015 (144)	1	13 years (post pubertal, pre-menarchal)	SCD	Prior to HSCT (busulfan/ cyclophosphamide/ ATG)	LSCO	POI	Required hormonal supplementation for menarche	- Desired pregnancy; discontinued HRT; FSH 59 IU/l, LH 32 IU/l) - 4 months after OTT: hormone levels FSH 5 IU/l; LH 6 U/l; estradiol E2 166 pg/ml) - 5 months after OTT: started regular menstruation - 2 years after OTT: conceived spontaneously; uncomplicated pregnancy *first case of premenarchal OTC resulting in pregnancy
Pecker et al., 2018 (139)	2	25, 27	SCD	Prior to HSCT	LSCO	NR	NR	1 patient experienced pain crisis after laparotomy
Armstrong et al., 2018 (145)	18 (114 total)	Range 2-13 years	10 SCD 8 BT	Unknown/prior to HSCT	NR	NR	NR	Not reported
Matthews et al., 2018 (146, 147)	1	9 years	BTM	Prior to HSCT	LSCO	Menarche age 15; sporadic menses - Patient trying to conceive 2 years; AMH <0.5ng/mL; FSH 18-67 IU/L	Started on estradiol/ Norgestrel for uterine development, regulation of menstrual cycle	- HRT discontinued after OTT - FSH and LH ranged from pre to post-menopausal range, at 5 mo AMH approached 2 ng/mL - Underwent IVF cycle (anatagonist), 8 oocytes retrieved from transplanted ovary; uncomplicated pregnancy

(Continued)

TABLE 3 Continued

Author, Year	Cases	Age	Diagnosis/ Genotype[1]	Indication for FPT	OTC	Post HSCT	Need for hormonal supplementation	Outcomes
Poirot et al., 2019 (148)	71 (418 total)	Range 0.3-15 years across all categories	"hemo-globinopathies" - not characterized	NR	NR	NR	NR	-oocytes isolated from the tissue were cryopreserved in 50 cases
Mamsen et al., 2021 (37)	14	Range 2.8-17.4 years	10 BT 4 SCD	NR	LSCO	Two patients underwent OTT-menopausal at time of transplant	NR	- follicle density, morphology, and expression of follicle- and oocyte specific proteins were comparable to an age-matched reference group - 3-4 months after OTT, serum hormone levels normal - 1 patient conceived with IVF, gave birth full term *first case of pre-pubertal follicle resulting in pregnancy
Dolmans et al., 2021 (149)	9 (285 total)	Range 9-44 years across all categories	"hemo-globinopathies" - not characterized	NR	NR	NR	NR	- All patients had OTT -40% spontaneous conception rate
Kristensen et al., 2021 (150)	1,186 total	Range 4mo-44 years across all categories	55 benign hematological disease	NR	NR	NR	NR	- 117 returned for OTT - Patients with benign hematological diseases had highest (35%) return rate
Hanfling et al., 2021 (151)	2	2, 18 years	1 SCD, 1 BT	Prior to HSCT (both on hydroxyurea)	LSCO	NR	NR	- Mature oocytes found at time of OTC
Boga et al., 2022 (114)	1	Not reported						

1- Diagnosis and genotype are those reported in manuscript
OTC, ovarian tissue cryopreservation; FPT, fertility preservation treatment; HSCT, hematopoietic stem cell transplant; OTT, ovarian tissue transplantation; SCD, sickle cell disease; BTM, beta thalassemia major; BT, beta thalassemia; POI primary ovarian insufficiency; NR, not reported; LSCO laparoscopic oophorectomy; ATG, anti-thymocyte globulin; E2, estradiol; HRT, hormone replacement therapy.

Spontaneous pregnancy may occur after OTC, although IVF is frequently required. In 2015, Demeestere et al., reported the first live birth from ovarian tissue cryopreserved from a pubertal female who was pre-menarchal. The OTC occurred at age 13. The patient, who had SCD, was confirmed to have primary ovarian insufficiency when she desired to conceive. Two years after ovarian transplantation, the patient spontaneously conceived (144). In 2021, Mamsen et al., reported the first case of pregnancy from OTC performed prior to puberty. The patient who had beta thalassemia underwent OTC prior to HSCT at 9 years old. She returned at 23 for OTT after which she had resumption of ovarian function and was able to conceive with IVF. In a separate case report, a patient required 14 cycles of IVF and 3 separate OTT to achieve a life birth (142). Unfortunately, most studies do not report on infertility rates among patients who have undergone OTT, nor do they report sufficient information on the indication for IVF or the number of cycles to draw conclusions about the chance of spontaneous conception after OTT. Furthermore, patients' response to HSCT is variable; some patients appear to be completely cured after treatment while others have a less robust response. Individuals who are not cured likely differ in pregnancy outcomes given the higher rate of stillbirth and fetal growth restriction in untreated patients with SCD (161).

OTC has dramatically altered opportunities for fertility preservation in pediatric patients, especially those who are prepubertal. Despite the promise of OTC, outcomes should be viewed cautiously. Globally, few pregnancies have occurred for patients who had OTC prior to puberty. While spontaneous pregnancies occur, they should not be viewed as expected. Furthermore, ovarian tissue grafts have a finite life. It is estimated that ovarian grafts last approximately 2.25 years on average (159). Therefore, periodic OTT may be required throughout a female's reproductive life.

Other fertility considerations

Women with SCD and beta thalassemia are at increased risks for obstetric complications including maternal mortality,

TABLE 4 Cases of controlled ovarian hyperstimulation in patients with hemoglobinopathies.

Author, Year	Age	Diagnosis	AFC	Days stimulated	Peak estradiol	Total Gonado-tropin IU/d	Trigger	#oocytes retrieved	#oocytes cryopreserved
Dovey et al., 2012 (160)	19 years	SCD	20	6	859	900	Leuprolide 20IU BID	9	8
Lavery et al., 2016 (154)	14 years	SCD	13	14	NR	2625	rHCG	7	7
	15 years	SCD	6	10	NR	1875	rHCG	5	4
	16 years	SCD	18	11	NR	131.5	rHCG	21	16
	16 years	SCD	16	10	NR	1462.5	rHCG	29	25
	16 years	SCD	16	10	NR	1500	rHCG	14	11
	17 years	SCD	20	11	NR	3350	rHCG	5	3
	18 years	SCD	20	10	NR	1875	rHCG	31	30
	18 years	SCD	12	12	NR	3075	rHCG	7	1
Matthews and Pollack, 2017 (146)	23 years	SCD	28	5	1,669	1,125	Leuprolide 80IU 2 doses at 36 and 24 hr	9	8
Pecker et al., 2018 (139)	26 years	SCD	2	13	3567	5850	Leuprolide	21	21
	28 years	SCD	Small follicles	10	244	3000	hCG	11	7 embryos
	32 years	SCD	10	10	983	1875	hCG	14	14
	28 years	SCD	Small follicles	12	815	3300	Leuprolide	4	3
	15 years	SCD	14	13	457	2925	hCG	14 (transabdominal)	12
Boga et al., 2022 (114)	1 patient had embryos cryopreserved, 3 had oocytes cryopreserved								

AFC, antral follicle count; SCD, sickle cell disease; NR, not reported; BID, twice daily.

intrauterine fetal demise, preeclampsia, preterm delivery, and spontaneous miscarriage (162–167). These risks are partly due to high rate of comorbidities associated with these hemoglobinopathies, i.e., hypercoagulability. For women with significant comorbidities who wish to have biologic children, the option for surrogacy should be discussed along with appropriate preconception counseling with maternal-fetal medicine specialists.

Another important component of fertility treatment is the discussion of genetic testing. Individuals who carry mutations for hemoglobinopathies should be offered preimplantation genetic testing (PGT) to reduce the risk of an affected offspring. While patients may not be ready for parenthood soon after their fertility preservation, education on surrogacy and PGT may be helpful in informing patients and their families on the full scope of fertility options.

Discussion

Preoperative and post-operative risk management

Both surgery and COH contribute to fluid shifts and hypercoagulability, which increase the risks for adverse outcomes among patients with SCD. It is estimated that 5% of pediatric patients experience postoperative VOC and ACS (168, 169), and moderate to severe OHSS occur in 1-5% of all COH cycles (138). To date, there have been six adverse outcomes reports from FPT: (Table 5): one episode of mild OHSS, four episodes of acute pain crises, and one episode of ACS requiring intubation and intensive care unit admission. All adverse events occurred in patients with SCD, and the more severe adverse events occurred in older patients with more comorbidities. Patients with BTM who have undergone a splenectomy are at increased risk of post-operative infections and those with hemosiderosis induced heart failure are more prone to fluid overload. However, these risks are less well described than for SCD. To date, no adverse events have been reported in pediatric SCD FPT or in any BTM patient undergoing FPT.

Preoperative and postoperative optimization are vital to reduce procedural complications and to reduce the risk of cancellation of high stakes cycles. While there are no standardized protocols for preoperative management, there are general principles which should be followed. Below, we discuss the available literature and include a protocol created by our center for management of COH in patients with SCD (Table 6).

Preoperative/preprocedural planning

Prior to FPT, coordination with the patient's hematologist is vital for procedural optimization and postprodcedural management. Universal preoperative anesthesia consult is not

TABLE 5 Adverse outcomes associated with fertility preservation treatment in patients with sickle cell disease.

Authors	Age	Type of FPT	Complication	Management
Dovey et al., 2012 (160)	19	COH	Acute pain crisis starting immediately post oocyte retrieval	Hospital admission
Lavery et al., 2016 (154)	18	COH	Mild OHSS 4 days post retrieval	Supportive care
Matthew and Pollack, 2017 (146)	23	COH	Acute pain crisis on day 6 of COH	Exchange transfusion; cycle continuation
Pecker et al., 2018 (139)	26	COH	ACS, respiratory failure, bacteremia	Intubation, intensive care unit admission, pain control, antibiotics
	27	OTC	Acute pain crisis	Red Cell exchange
	28	COH	Acute pain crisis on day 6 of COH	Hospital admission; IV hydration; pain control

FPT,-fertility preservation treatment; COH,- controlled ovarian hyperstimulation; OTC,-ovarian tissue cryopreservation; OHSS,-ovarian hyperstimulation syndrome.

warranted (170) but should be considered based on patient's medical comorbidities. Ensuring that children are up to date on disease-specific screening (i.e. transcranial Doppler ultrasound) is also recommended, although this is expected if the patient is in the process of undergoing HSCT. Patients may have a history of prior VTE, and thus an anticoagulation plan must be considered when planning for both surgery and COH. To reduce overall surgical risks, coordinating with other procedures, such as port placement, should be considered.

Creating a COH stimulation protocol that optimizes oocyte yield while minimizing risk of OHSS is vital. In our practice, we use either hCG or a gonadotropin-releasing hormone agonist for trigger to reduce the risk of OHSS (139). Given the unclear impact of hydroxyurea on ovarian reserve, oocyte quality and embryo development (171), we also recommend discussing medication discontinuation prior to FPT.

Data on the benefits of preoperative transfusion in patients with SCD is conflicting (172–176). Nevertheless, most experts recommend transfusion for a hemoglobin level ≥9-10 g/dL and exchange transfusions for hemoglobin S <30% (170, 177), and these are the benchmarks that our group has recommended for management of COH (139). For patients who receive regular transfusions or exchanges, these should be continued in the immediate preoperative period. If transfusion or exchange is planned, coordination with blood bank specialists who are familiar with the patient's transfusion history and have knowledge of any red blood cell autoimmunization or alloimmunization is recommended (139). Decisions on if and when transfusions or exchanges are recommended should be discussed with the patient's hematologist.

Perioperative

Triggers for sickling should be minimized in the perioperative setting, including dehydration, acidosis, hypoxia, and hypothermia. To prevent dehydration, it is recommended that patients avoid prolonged fasting, consume clear liquids up to two hours prior to their procedure, and receive IV hydration while fasting. Our group recommends administering IV hydration prior to anesthesia administration (139). Normothermia through use of body temperature monitoring systems, blankets, and ambient temperature control is recommended. Monitoring oxygen saturation is paramount, and supplemental oxygen used when indicated. Glucocorticoids, such as dexamethasone, should be avoided as they may precipitate pain crises (139, 178, 179).

Postoperative

Use of incentive spirometry, chest physiotherapy, and early ambulation in the postoperative period is widely recommended to reduce the risk of ACS (139, 170). Early pain control is another important facet to postoperative management. Patients with chronic pain, such as in SCD, may have altered pain perception and may already be taking daily narcotics.

TABLE 6 Perioperative considerations.

Preoperative	Perioperative	Post-operative
- Coordination with anesthesia and hematology - Develop pain management plan in coordination with hematology - Consider exchange transfusion - Consider HU discontinuation - IF HU naïve and planning on starting HU prior to HSCT, perform FP prior - Consider prophylactic anticoagulation if history of VTE	- First or early start case - Clear liquids up to 2 hours prior to start time - IV hydration when fasting and prior to anesthesia - Minimizing hypothermia in pre-operative space, in the operating room, and in recovery - Avoidance of dexamethasone for nausea	- Early pain management - Early incentive spirometry - Discontinue IV hydration when tolerating by mouth

HU, hydroxyurea; HSCT, hematopoietic stem cell transplant; FP, fertility preservation VTE, venous thromboembolism; IV, intravenous

Preoperative discussion with the patient's hematologist is absolutely critical when determining postoperative pain regimen. Narcotics are often first line agents, although patient-controlled analgesia may be warranted (170). Understanding the patient's recent pain history may help to predict postoperative complications, as patients with recent hospitalizations for crises are more likely to have postoperative crises (180).

Health care and research disparities

Hemoglobinopathies are the most commonly inherited monogenetic diseases, yet research and funding do not reflect the prevalence of the diseases (125, 181). For example, sickle cell related variables are not collected in large health outcomes databases and no robust dataset exists for hemoglobinopathies and fertility (28), thereby limiting providers' ability to offer evidence based care.

No studies to date have evaluated access to FPT care for patients with hemoglobinopathies (125). However, fertility preservation is not commonly utilized prior to HSCT. For example, in a claims database study of over 400 adults who underwent HSCT, only 7% had claims for fertility preservation services before their transplant (182). A significant barrier to care, especially in the adolescent population is timely referrals. Pediatric providers may not be aware of infertility risks and may feel poorly equipped to discuss fertility preservation and uncomfortable discussing reproductive health with patients and their families (183–185), especially with rapidly evolving recommendations and practices.

For patients who do receive a referral for fertility preservation, the cost of FPT may be a significant barrier. The average cost of COH for fertility preservation is over $12,000 (186, 187), and the cost of laparoscopic oophorectomy is comparable (186). Storage fees for cryopreserved oocytes and ovarian tissue, which may be over twenty years, further adds to required costs. Whereas programs such as Livestrong exist to assist patients in fertility preservation prior to cancer treatment, no such national program exists for hemoglobinopathies. As of 2022, 12 states mandate coverage for fertility preservation prior to gonadotoxic treatment (188). However, mandates often do not cover government assistance such as Medicaid and Medicare, and some states such as Utah require that the patient has a cancer diagnosis, thereby disqualifying many patients with SCD or BTM. Some have argued for the need to change institutional programs (125) to provide coverage for patients with SCD. Ultimately, advocating for legislation change both on the local and national level is needed to expand coverage for this population.

Adoption and utilization of oncofertility patient navigators (189) may help to reduce some of these barriers to fertility preservation among patients with hemoglobinopathies. Patient navigators help guide patients and their families through fertility preservation, from identifying an in-network clinic, expediting fertility evaluation, providing education about different fertility options, and referring to different support groups (190). Through advocating for patients and their families, navigators may play a vital role in empowering them to make the right fertility preservation decisions for their circumstances and goals.

Conclusions

Sickle cell disease and beta thalassemia are the most common and morbid hemoglobinopathies. Disease modifying and curative treatments have improved quality of life and increased the chance of living into adulthood. However, many of these treatments negatively impact fertility and normal pubertal development. Fertility preservation should be discussed with all patients and families considering disease modifying and curative therapies. In very young children in which fertility preservation may be challenging, the risks and benefits to delaying HSCT for greater maturation should be discussed. Counseling patients and families about future fertility must take into consideration the patient's disease, treatment history, and planned treatment, acknowledging current knowledge gaps. Preparing for fertility preservation must also include a multidisciplinary approach to optimize patient outcomes while reducing surgical and procedural risks. Further research and advocacy are needed to improve patient care and future fertility.

Author contributions

MC, BB, LP and TK contributed to conception and outline of the manuscript. BB wrote the first draft of the manuscript. TK, LP and MC wrote sections of the manuscript. All authors contributed to manuscript revision, read, 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

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EDITED BY
Mahmoud Salama,
Michigan State University,
United States

REVIEWED BY
Nalini Mahajan,
Independent researcher, New Delhi,
India

*CORRESPONDENCE
Dunja M. Baston-Büst
dunja.baston-buest@med.uni-duesseldorf.de

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Fertility preservation in the pediatric population—experience from a German Cryobank for ovarian tissue

Dunja M. Baston-Büst* and Alexandra P. Bielfeld

Department of OB/GYN and REI, UniKiD/UniCareD, Medical Hospital University of Düsseldorf, Düsseldorf, Germany

Counseling children on the possibility of fertility preservation prior to a gonadotoxic treatment supports the decision-making process, taking into account that the patients are in a very vulnerable and mentally exhausting situation following the diagnosis. Referral to specialists can be optimized on-site by routing slips with contact addresses, phone numbers, and mail contacts; available time slots for consultation; possibly offers for cost coverage; and an easy-to-understand information leaflet about the different options available. Some of the options for fertility preservation in the prepubertal population especially are still experimental. The unique possibility of fertility preservation before the onset of the gonadotoxic therapy, which may cause premature ovarian insufficiency or azoospermia in the future, should be highlighted.

KEYWORDS

cryopreservation, freezing, juvenile, children, infertility

Introduction

Cancer registries

The cases of children suffering from pediatric cancer pathologies are listed in cancer registries worldwide. The first challenge is that the registers are not able to represent all cases; e.g., the German register for pediatric cancer lists an estimated 95% of all cases (https://www.kinderkrebsregister.de/dkkr/ueber-uns/uebersicht.html). From the beginning of data acquisition in the 1980s in Germany, when the registry was founded in a University setting, a worldwide connection (e.g., the International Association of Cancer Registries or the European Network of Cancer Registries) was pursued to present a comprehensive data set. A further challenge is that the data acquisition ends at the patient age of 18.

Childhood cancer and infertility

Due to the aforementioned limitations in data collection, patient follow-up is proving difficult. For example, cases of premature ovarian failure (POF) in adolescent female cancer survivors or azoospermia in adolescent male cancer survivors cannot be linked to the type of cancer or the treatment that was received during childhood. Thirteen out of 30 female patients counseled in our center for reproductive endocrinology and infertility (UniKiD) due to POF are former pediatric cancer treatment patients. These female patients reported the cancer treatment they received during childhood in the medical history questionnaire. Most of these patients did not suspect that the POF resulted from the treatment of their childhood cancer. A recent study on the use of ART by childhood cancer survivors highlights the higher incidence of ART treatments compared with general ART statistics in Germany. Fresh cycle oocytes or sperm were mainly used during ART treatment (1). There were more multiples born and a higher prevalence for low birth weight in the offspring of cancer survivors, whereas the prevalence for preterm birth or small for gestational age were comparable to spontaneously conceived offspring of cancer survivors. Neither childhood cancer nor congenital malformations were found to be increased in the offspring (1). Therefore, it is extraordinarily important that every patient of reproductive age and every child should have the possibility to be counseled in a specialized center by gynecologists, reproductive/endocrine specialists, or urologists/andrologists before the gonadotoxic treatment starts as recommended by the current S2k guideline on fertility preservation for patients with malignant diseases (2). During counseling, the risks of infertility as a result of the gonadotoxic treatment (chemotherapy or surgery or radiation), the possibilities for fertility preservation, the risk of metastasis for systemic diseases, and the individual case should be discussed (3). There should also be a follow-up during puberty or if the onset of puberty takes place after the age of 14 with hormone analyses, ejaculate analyses, and Tanner scoring as recommended by endocrinologists. All data of primary or secondary amenorrhea or azoospermia should be sent to the registries, keeping in mind the lack of registries after the age of 18 (2).

Options of fertility preservation in the prepubertal child

Fertility preservation in the young child is limited due to the outstanding puberty and physical immaturity. The freezing of ovarian tissue biopsies is the only option for young girls and the only promising option for females if less than 14 days remain until the beginning of the gonadotoxic therapy (4, 5). Concerning young boys, the techniques are even still more experimental and part of current research (6, 7). The cryopreservation of several small pieces of the immature testicular tissue and the isolation and subsequent freezing of spermatogonial stem cells (SSCs) can be performed in specialized cryobanks using stem cell freezing protocols. For a long time, techniques concerning later transplantation or *in vitro* maturation for tissues or cells of young girls as well as boys have been experimental. Besides the ethical discussion regarding the use of these biopsies and the possible dissemination of malignant cells, the techniques still need to be proven in the clinical routine for childhood cancer survivors (8–10). There are only a few case reports of ovarian tissue cryopreservation (OTC) during childhood, transplantation in the adolescent female and subsequent successful pregnancies and deliveries (11, 12). OTC in the adult female and transplantation is an already accepted option (13–16). *In vitro* maturation of immature oocytes from prepubertal females harvested during the OTC procedure showed a lower maturation potential compared with oocytes from adult females (17). Recently, the first deliveries were reported after *in vitro* maturation of oocytes collected at the time of OTC and subsequent vitrification (18). The transplantation techniques for male childhood cancer survivors are still more experimental (19–21). Some of the options include grafting or injection of the thawed SSCs in the remaining testis, *in vitro* models like testicular organoids, or *in vitro* growth and differentiation (22, 23). Full spermatogenesis after grafting is demonstrated in several animal models (24–27). The success of grafting and other methods still seems to be linked to the maturation state of the donor testis (prepubertal, pubertal, or adolescent) even keeping in mind that animal models have a shorter life span and shorter time of puberty. Mimicking the puberty of the male *in vitro* is still challenging (23, 28).

Options of fertility preservation during and after puberty

Besides the possibilities for prepubertal children (OTC and SSC, respectively), there are more options in the older child, especially for boys. Young males during and after puberty are more likely able to ejaculate. Motile sperm of those samples can be frozen according to state-of-the-art protocols of the IVF or andrology/urology lab using slow freezing or vitrification before the gonadotoxic therapy starts (29–31). If the ejaculated sample does not contain enough sperm, the child can be counseled for testicular tissue cryopreservation (4). Young female patients during puberty under the age of 18 have a contraindication for controlled ovarian stimulation due to their age because the medication is not licensed for this age group in Germany. Young females can be counseled for an off-label use to

combine both techniques, OTC and freezing of mature oocytes. Recent approaches even combine OTC and *in vitro* maturation of immature oocytes (32, 33).

As a second option, gonadotropin-releasing hormone agonists (GnRHa) can be offered to female patients after puberty (2). Unfortunately, the results of the meta-analysis of the application of GnRHa are conflicting because different endpoints were examined, e.g., the prevalence for premature ovarian insufficiency, the duration of amenorrhea, or pregnancy rate (2).

Counseling young patients with cancer

In Germany, counseling of a young patient with cancer and/or before a gonadotoxic therapy starts is considered necessary for the patient. Therefore, patients can make a comprehensive decision as part of the overall therapy strategy. Hence, the obligation to offer advice is described in the national guidelines (2). It also needs to be taken into account that children are not legally allowed to make a decision, which means that the child's parents need to be counseled along with the child. The aim of counseling intervention is to support the decision-making process and reduce possible decisional conflicts and anxiety and also to depict a strategy for the future. The decision to undergo fertility preservation may be affected by a multitude of psychosocial factors. Most patients who are referred for fertility preservation counseling are in the early stages of coping with their cancer diagnosis. The patients may be struggling with their mortality, future recurrences, and illness-related sequelae (34–36). Additionally, the preexisting anxiety regarding the illness itself can be intensified by the time sensitivity of the decision (37). Another burden in counseling is the financial burden to the child or the child's family when pursuing fertility preservation because most insurance policies do not cover the treatment costs. Besides all these issues, it is widely accepted that patients benefit from counseling depicting choices and, therefore, making them visible and relevant (38, 39).

The burden of costs for fertility preservation

Health insurance companies unfortunately do not cover the costs for fertility preservation in the pediatric population in general. We need to consider that the time of storage of the OT or SST might be up to 30 years until the former children want to start a family. Regulations concerning cost coverage of fertility preservation vary worldwide and change constantly. Since July 2021 in Germany, the costs for MII freezing in patients >18 years old are covered by health insurance when, e.g., the mammary cancer is not hormone receptor–positive. Oocyte freezing has been covered in the state of New York for medical reasons since March 2021. Freezing of ejaculated sperm or TESE biopsies is already covered in pubertal male patients. The costs of cryopreservation in female patients <18 years old and in pre/pubertal male patients can be supported by foundations or clinical studies if applicable. Some cryobanks offer freezing of SSC for free as long as the procedure remains within an experimental status.

The network Fertiprotekt

In German-speaking countries, the network Fertiprotekt (https://fertiprotekt.com/) collects national data for cryopreservation of cells and tissue of female patients. The network was founded in 2006, and more than 150 centers located at universities or private settings are voluntary members by now. The data are published annually as part of the national IVF registry (https://www.deutsches-ivf-register.de/). In 2020, more than 1500 female patients were counseled regarding their options for fertility preservation (https://www.deutsches-ivf-register.de/perch/resources/dirjb2020en.pdf) and about 1000 decided to perform some kind of preservation option, including GnRHa. Approximately 60 young female patients under 15 years of age were counseled and about 160 young females between 15 and 20 years old. In 2020 in total, 327 OTCs were performed in German-speaking countries.

Own data

Getting more into detail for the female pediatric patient, we now present our own data for this particular group. Between 2018 and May 2022, OTCs of 104 girls with a mean age of 14 years (range 1–17 years) were frozen at the UniCareD Cryobank in Düsseldorf. These girls and their parents were counseled in different centers, universities, and hospitals in their pediatric oncology or hematology departments within Germany. The surgery was performed in either the pediatric surgery unit of the university hospital in Düsseldorf or a surgery unit near their hometown with overnight shipping in specialized boxes. All the referring centers have signed cooperation contracts. As soon as the girl and her parents agree to the option of cryopreserving OT, the centers announce the surgery before the start of the gonadotoxic treatment. A special transport box with cooling packs that keep the temperature between 4°C and 8°C for 24 h is offered from the UniCareD within 24 h after announcement. The overnight shipping is performed between 4°C and 8°C in an organ transport medium (Custodiol ®, Dr. Franz Köhler Chemie GmbH, Bensheim, Germany). According to our checklist, the centers keep the cooling packs and the tube filled with Custodiol in a fridge on-site. All surgeons are advised to observe a further

piece of the ovary for possible metastasis in the pathology unit on-site. As part of our setup, we also assessed 3 × 2 mm ovarian biopsies before cryopreservation to assume a vitality score (follicle count) after enzymatic digest with collagenase and fluorescent staining with calcein AM (both Merck KGaA, Darmstadt, Germany). This follicle count ranged from 1 to 1000 follicles per 3 × 2 mm biopsies with a mean of 202 (median 160). This follicle count can be used to plan the number of cortex pieces to transplant later. The cryopreservation process in the UniCareD starts with the preparation of the cortex within a class A hood on a cool plate. The cortex pieces are frozen according to a slow freezing protocol with automatic seeding in multipurpose handling medium (MHM, Fujifilm Irvine Scientific, Santa Ana, CA, USA) supplemented with DMSO as a cryoprotectant. Long-term storage of the samples is performed in the gas phase in a liquid nitrogen tank. Focusing on the pathologies, 1/3 of these patients suffered from a Hodgkin lymphoma, 1/10 from Ewing sarcoma, and <1/10 from osteosarcoma and ß-Thalassemia during initial diagnosis. Two patients were counseled with a recurrent malignancy. All physicians and the patients were informed about the results of the vitality test, the number of pieces of the ovarian cortex frozen (ranging between 3 and 10), and the long-term storage of the samples.

Discussion

The number of cancer survivors who suffer from either childhood or adolescent cancer is rising due to the latest developments in chemotherapies by using new schemes or targeted therapies. Every patient of reproductive age and every child should have free access to an oncofertility consultation prior to gonadotoxic therapy. There should be no financial burden or lack of a time slot. Centers specialized in the counseling and treatment of patients at risk for fertility loss can try to optimize their work, e.g., with a specialized team for fertility preservation, dedicated phone number or mail contacts, standard operating procedures on-site, and networking at their location or nationwide (e.g., Fertiprotekt). Propagating all the information about the options for fertility preservation on the website of the specialized centers will improve the referral of juvenile as well as adolescent patients at risk. As it stands, the referral rate to an oncofertility unit in Germany for female patients diagnosed with cancer is below 10%, unfortunately (40). Health care providers and physicians of different disciplines can be offered more information, and legal aspects can change, e.g., the cost coverage of MII oocytes for medical reasons in different countries worldwide. Acquiring data on the incidence of cancer, the performance of fertility preservation techniques, freezing conditions, storage, and the later use of the tissue or cells is necessary to improve the outcome for the patients, especially for childhood cancer survivors with partially experimental techniques. One of these aims is to fully grow and mature oocytes *in vitro* from strips of ovarian tissue for patients at high risk of reintroduction of malignant cells by retransplantation (41, 42). Furthermore, the outcome for female patients could be optimized by maturation of immature oocytes aspirated during the endoscopic biopsy of ovarian tissue (43). Thawing and transplantation techniques for male patients need to be refined, but in our opinion, research is progressing, and male grafting or *in vitro* differentiation might possibly be realized in 5 or 10 years.

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

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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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EDITED BY
Asma Chattha,
Mayo Clinic, United States

REVIEWED BY
Qinjie Tian,
Peking Union Medical College Hospital (CAMS), China
Ani Amelia Zainuddin,
National University of Malaysia, Malaysia

*CORRESPONDENCE
Monica M. Laronda
mlaronda@luriechildrens.org

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Differences in gonadal tissue cryopreservation practices for differences of sex development across regions in the United States

Aisha L. Siebert[1,2], Veronica Gomez-Lobo[3], Emilie K. Johnson[2,4], Leena Nahata[5], Kyle E. Orwig[6], Louise C. Pyle[7], Selma F. Witchel[8], Courtney Finlayson[9] and Monica M. Laronda[1,10]*

[1]Stanley Manne Children's Research Institute, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, United States, [2]Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL, United States, [3]Pediatric and Adolescent Gynecology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, United States, [4]Division of Urology, Ann & Robert H. Lurie Children's Hospital, Chicago, IL, United States, [5]Department of Pediatrics, Pediatric Endocrinology, The Ohio State University College of Medicine, Columbus, OH, United States, [6]Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States, [7]Roberts Individualized Medical Genetics Center, Division of Human Genetics and Department of Pediatrics, Perlman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [8]Division of Pediatric Endocrinology, Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States, [9]Division of Endocrinology, Department of Pediatrics, Ann & Robert H. Lurie Children's Hospital, Chicago, IL, United States, [10]Division of Endocrinology, Department of Pediatrics, Feinberg School of Medicine, Northwestern University, Chicago, IL, United States

Objective: Some individuals with differences of sex development (DSD) conditions undergo medically indicated prophylactic gonadectomy. Gonads of individuals with DSD can contain germ cells and precursors and patients interested in future fertility preservation and hormonal restoration can participate in DSD-specific research protocols to cryopreserve this tissue. However, it is unclear how many providers or institutions offer gonadal tissue cryopreservation (GTC) and how widespread GTC for DSD is across the United States (US). The Pediatric Initiative Network (PIN) and Non-Oncologic Conditions committees of the Oncofertility Consortium sought to assess the current state of GTC for patients with DSD.

Methods: An electronic survey was sent to providers caring for patients with DSD *via* special interest groups of professional societies and research networks.

Results: The survey was administered between November 15, 2021 and March 14, 2022. A total of 155 providers responded to the survey, of which 132 respondents care for patients with DSD, and 78 work at facilities that offer medically indicated gonadectomy to patients with DSD diagnoses. They represented 55 US institutions including 47 pediatric hospitals, and 5

international sites (Canada, Denmark, Germany, Qatar). Of individual providers, 41% offer cryopreservation after prophylactic gonadectomy for patients with DSD (32/78). At an institutional level, GTC after medically indicated gonadectomy is available at 54.4% (24/46) of institutions. GTC is offered for a variety of DSD diagnoses, most commonly 45,X/46,XY DSD (i.e., Turner Syndrome with Y-chromosome material and mixed gonadal dysgenesis), ovotesticular DSD, complete androgen insensitivity syndrome (CAIS), and complete gonadal dysgenesis. Responses demonstrate regional trends in GTC practices with 83.3% of institutions in the Midwest, 66.7% in the Northeast, 54.6% in the West, and 35.3% in the South providing GTC. All represented institutions (100%) send gonadal tissue for pathological evaluation, and 22.7% preserve tissue for research purposes.

Conclusions: GTC after gonadectomy is offered at half of the US institutions represented in our survey, though a minority are currently preserving tissue for research purposes. GTC is offered for several DSD conditions. Future research will focus on examining presence and quality of germ cells to support clinical decision making related to fertility preservation for patients with DSD.

KEYWORDS

differences of sex development (DSD), intersex, gonadal tissue cryopreservation, fertility preservation, oncofertility

1 Introduction

Individuals with disorders/differences of sex development (DSD) conditions, also termed intersex, experience atypical or discordant phenotype of the external genitalia in relation to the chromosomal or gonadal sex. DSD encompasses a broad range of diagnoses with variable phenotypes and malignancy risks. The indications and timing of prophylactic gonadectomy vary for individual DSD diagnoses. There is a paucity of clinical practice guidelines, but literature reviews are available on the topic of timing and indication for gonadectomy by DSD diagnosis (1, 2). Tissue removed during medically indicated gonadectomy for malignancy prevention may have future therapeutic value for fertility preservation or hormonal restoration (3).

Research on tissue maturation for future fertility or hormonal restoration is in progress, with the greatest experience coming from typical ovarian and testicular tissue. Prepubertal ovarian tissue cryopreservation (OTC) initially emerged as an experimental option for fertility preservation prior to gonadotoxic cancer therapies. In 2019 the American Society for Reproductive Medicine (ASRM) removed the experimental label because of the more than 140 reported live births following ovarian tissue transplantation of cryopreserved tissue (4–6). However, research is still ongoing, especially for prepubertal individuals undergoing OTC (7). Prepubertal testicular tissue cryopreservation (TTC) is considered experimental, but primate studies have demonstrated feasibility of grafting with return of spermatogenesis and subsequent live birth (8). Providers caring for patients with DSD may offer cryopreservation of excised gonadal tissue, or gonadal tissue cryopreservation (GTC), under a research protocol, in the hopes that current OTC and TTC maturation studies, and ongoing research into the type of cells present within gonads of different DSD diagnoses will benefit patients with DSD in the future.

Largely in response to advocacy from families and patients with DSD diagnoses, in 2018 the first US based research template for GTC in DSD was initiated (9). GTC is categorized as experimental because how the tissue may be used to offer options for biological offspring or hormone restoration has not yet been elucidated (10). It is important to note that there have been no published reports of transplantation of gonadal tissue following GTC in DSD populations, nor have any mature gametes been isolated from tissue that has been cryopreserved from these individuals. Cryopreserved gonadal tissue would most likely be used with advanced reproductive technologies (ART), and before it can be applied clinically successful demonstration of *in vitro* maturation of immature oocytes and *in vitro* spermatogenesis is necessary.

It is unclear how many providers or institutions have access to GTC for DSD, either at their own institution or through an inter-institutional agreement. To assess the current state of GTC

for patients with DSD diagnoses – specifically rates of GTC after medically indicated gonadectomy for tumor prevention – as well as to inform future DSD fertility research, the Pediatric Initiative Network (PIN) and Non-Oncologic Conditions committees of the Oncofertility Consortium queried providers caring for these patients. *The aim of this survey was to assess national trends in GTC in the setting of medically indicated gonadectomy for patients with various DSD diagnoses.*

2 Material and methods

A notice of exemption for this study was obtained from the Ann & Robert H. Lurie Children's Hospital of Chicago Institutional Review Board (IRB). The survey was administered to providers caring for patients with DSD between November 15, 2021 and March 14, 2022. Study data sheet and a weblink to an online Qualtrics Survey were sent out *via* the email listservs for the following organizations: American Academy of Pediatrics Special Interest Group for Clinical Geneticists; American Society for Reproductive Medicine Fertility Preservation Special Interest Group; Disorders/Differences of Sex Development – Translational Research Network; National Society of Genetic Counselors; North American Society for Pediatric and Adolescent Gynecology; Oncofertility Consortium Pediatric Initiative Network & Non-oncologic Conditions; Pediatric Endocrine Society DSD Special Interest Group; Society for Pediatric Urology; and the Society for the Study of Male Reproduction.

Participation in the study was completely voluntary, and all potential participants were provided with a study information sheet and consented to the use of response data in a descriptive summary of national trends. Potential respondents were screened, and only providers who self-identified as caring for patients with DSD were invited to complete the full survey. Respondents completed a 12-item survey developed by the multidisciplinary research team, consisting of multiple-choice and free text responses enquiring about gonadal care, including gonadectomy and GTC practices by individual DSD diagnoses (Supplemental Table 1). The online survey took approximately five minutes to complete.

3 Results

3.1 Many individual providers and institutions offer GTC for DSD diagnoses

A total of 155 providers initiated the survey and 132 respondents indicated that they care for patients with DSD. Because lists of subscribers to the listservs used to distribute the survey were not shared, the response rate could not be calculated. Fifty-two responses were excluded because they did not provide an email address or institution and therefore could not be verified as a unique responder. Respondents represented 55 US institutions including 47 pediatric hospitals, and 5 international sites with two facilities in Canada, and one each in Denmark, Germany, and Qatar. The majority of responses were from US based providers with up to five responses from a single institution.

Of respondents who completed the full survey, 97.5% (78/80) individual providers offer medically indicated gonadectomy for patients with DSD, of which 41% (32/78) offer cryopreservation of excised gonadal tissue. GTC is available at 54.4% (25/46) institutions represented in the survey that reported on availability of this procedure. Only 9 providers indicated age at which cryopreservation is offered, collectively indicating a range of 0 to 100 years, with the average minimum age of 5.7 years and average maximum age of 25.8 years. Practice patterns of multiple providers at the same institution demonstrated a high degree of concordance in survey responses, with only two institutions in which one provider indicated "Don't know" and another provided diagnosis and/or age range information. In such cases, the more complete response was used for that institution

3.2 Institutional practice patterns were assessed by US census region

To evaluate regional differences, responses were aggregated by institution and analysis was performed on the US subset of represented institutions. To assess trends by geographic location, the proportion of institutions offering GTC was examined across US census regions. The highest percentage of facilities offering GTC were from the Midwest 83.3% (5/6), followed by 66.7% (6/9) in the Northeast, 54.6% (6/11) in the West and 35.3% (6/17) in the South (Figure 1). Although the observed differences were not statistically significant (Fisher exact test, p=0.77), we did note that the regions with the largest number of institutions represented in our survey were the least likely to offer cryopreservation. We examined institute or facility size as a possible confounder of the observed regional differences. The proportion of institutions offering GTC were not different by number of hospital beds (Fisher exact test, p>0.99) (Figure 2).

One hundred percent (44/44) of the institutions that report offering gonadectomy, and which provided information on tissue applications send gonadal tissue for pathological evaluation. Only 22.7% (10/44) of institutions preserve tissue for research purposes, including work to advance future therapeutic application of cryopreserved tissue (Figure 3). Of institutions offering GTC and reporting on where tissue it

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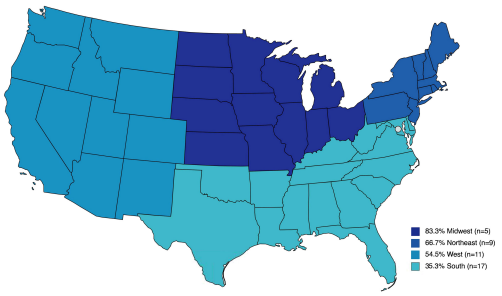

FIGURE 1
Percent of institutions that participated in the survey and offer gonadal tissue cryopreservation by United States census region.

cryopreserved, 55% (11/20) perform tissue freezing on site and the remaining 45% (9/20) ship samples to another center for cryopreservation.

3.3 GTC is offered for all surveyed DSD diagnoses

GTC is offered at participating institutions for a variety of DSD diagnoses following medically indicated gonadectomy, most commonly 45,X/46,XY DSD (66.7%), followed by CAIS (62.5%), Ovotesticular DSD (54.2%), and PAIS (50%). Patients with partial (45.8%) and complete gonadal dysgenesis (41.7%) are least likely to be offered cryopreservation (Figure 4).

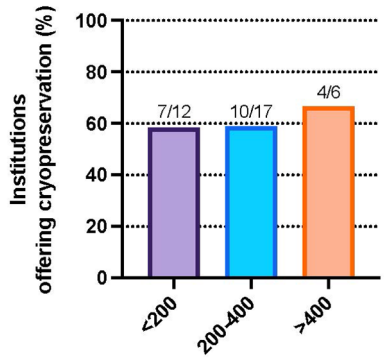

FIGURE 2
Percent of institutions that participated in the survey and offer gonadal cryopreservation for DSD patients by facility beds, <200 beds (58.3%), 200-400 beds (58.8%), and >400 beds (66.7%), p=NS.

4 Discussion

Our survey showed over a third of DSD providers who work at institutions that perform gonadectomy for DSD report offering GTC for this population. Gonadectomy was most commonly offered for conditions with the greatest malignancy risk including 45,X/46,XY DSD, complete and partial gonadal dysgenesis, and PAIS. GTC was offered most commonly for 45, X/46,XY DSD, CAIS, ovotesticular DSD, and PAIS. Respondents are least likely to offer cryopreservation after gonadectomy for partial and complete gonadal dysgenesis.

Understanding the current state of GTC for DSD is important because prior research indicates that future fertility is a concern for patients with DSD, who are open to many family-building options and desire autonomy in their decision making (11, 12). To be responsive to patient and family needs, a GTC for DSD research protocol has been developed that shows that pathologic evaluation of half of the gonad can be safely used to assess tumor burden and borders as well as presence of germ cells following medically indicated gonadectomy. If no tumor is present, the remaining half of the gonad can be cryopreserved for long term storage and potential future fertility or hormone restoration (9). Individual patients may also elect to donate a small portion of tissue to ongoing research. Though this concept was published from one institution, the present study indicates that GTC for DSD is being operationalized at 25 institutions in the US while less than a third of those engaging in research to advance clinical applications of that tissue.

A retrospective study of gonadal biopsy specimens from patients with DSD showed germ cells in some gonads from patients who previously would have been assumed to be infertile (e.g., streak ovaries, dysgenetic gonads, ovotestes), particular for the youngest patients aged 0 to 3 years old. (13). Additional research that identifies the type of germ cells present, their point in mitosis or meiosis, the type of supportive somatic cells and the quality of resulting gametes must be performed to assess the restorative potential of these germ cells. This research will add to the personalized care of this heterogenous patient populations that fall under the umbrella term of DSD, and may provide further understanding of the mechanisms of gonadal sex development. Through our recent survey, we now have a better appreciation for the scope of GTC availability for DSD, which will facilitate future collaborative studies.

We mapped the number of institutions that offered GTC across the US. In our survey, participating institutions in the Midwest were most likely, whereas institutions in the South were least likely to offer GTC. Individual provider and institutional responses that care for DSD patients spanned the continental US and there was no difference in the size of the facility that indicated the likelihood of a facility to offer GTC. There is a paucity of information assessing future fertility potential of cryopreserved gonadal tissue for individuals with DSD. Practice responses in our survey span all size institutions and

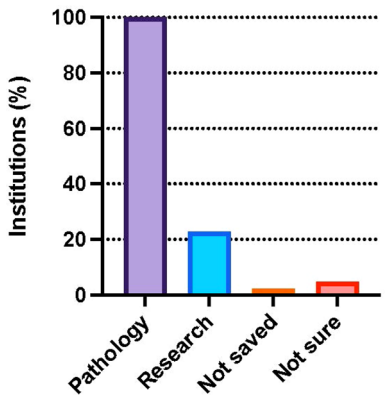

FIGURE 3
Percent of institutions that participated in the survey and perform pathology (100.0%) and research (22.7%) on tissue isolated from DSD patients. Few institutions reported tissue is not saved (2.3%) or were unsure (4.5%). Survey participants could choose more than one response.

almost half of represented institutions that offer GTC utilize an inter-institutional agreement for this service. There is a great opportunity for research on a larger scale through inter-institutional collaborations to inform future clinical care for DSD diagnoses.

4.1 Limitations

Our population lacks a denominator estimate, therefore making it impossible to assess response rate. We surveyed membership of 12 different organizations to which providers caring for patients with DSD may belong and we cannot determine the degree of overlap between these large listservs, the number of their membership who care for patients with DSD diagnoses, nor the number of providers who care for individuals with DSD but do not belong to these professional societies or subscribe to their listservs. Additionally, the number of providers or institutions who offer GTC are based on respondent reports and were not independently verified by chart review. Respondents provided aggregate reporting of cryopreservation practices by diagnosis as well as limited information on age at which gonadal care services are performed for various DSD diagnoses. Observed trends may reflect either differences in practice patterns, sampling bias, or a combination of the two. Cost of GTC varies by circumstance and insurance and may significantly impact access. In our experience the average cost of GTC is $1000 per patient when surgical cost is bundled with other oncologic care (14). Additional follow-up studies are needed to understand indications and timing of GTC following medically indicated gonadectomy for patients with various DSD diagnoses in the United States.

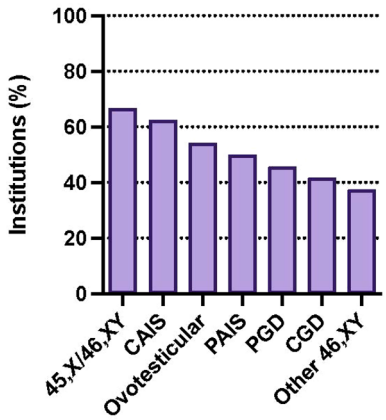

FIGURE 4
Percentage of institutions offering GTC by diagnosis: 45,X/46,XY DSD (66.7%), CAIS (62.5%), ovotesticular DSD (54.2%), PAIS (50%), partial gonadal dysgenesis (PGD) (45.8%), complete gonadal dysgenesis (CGD) (41.7%). Survey participants could choose more than one response.

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

AS adapted the survey to an online format, compiled responses, analysed data, and prepared results for publication. EJ, CF, and ML developed the survey content. AS, EJ, VG-L, LN, KO, LP, SW, CF, and ML distributed the survey *via* professional organization listservs and provided critical input on survey data interpretation. 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.990359/full#supplementary-material

SUPPLEMENTARY TABLE 1
Gonadal Care Survey text

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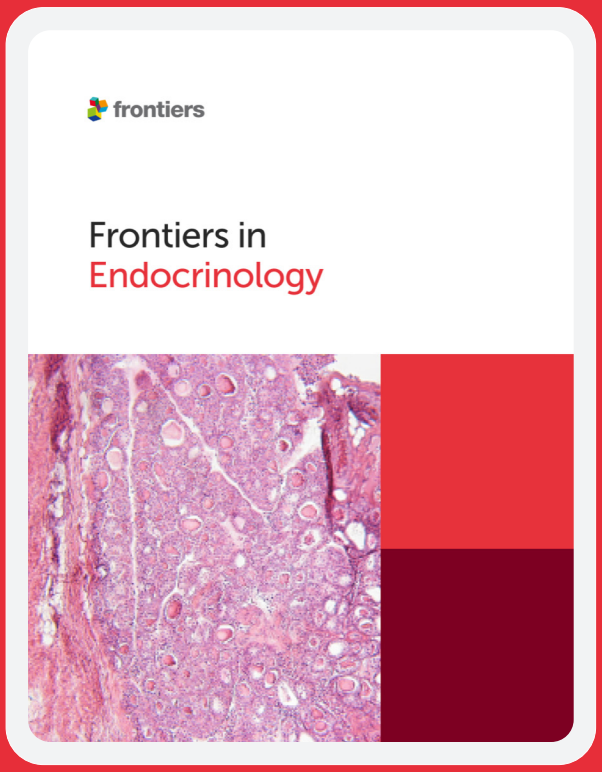

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