Title	Successful freezing of sperm cells via a testicular sperm extraction (TESE) procedure or semen cryopreservation in transfeminine adolescents with gender dysphoria
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Research proposal

Rationale	Gender dysphoria is the experience of significant dissatisfaction with one's assigned sex at birth, which does not align with one's gender identity. Gender dysphoria is associated with mood swings, anxiety disorders, and suicidality. In transgender and gender diverse individuals, gender identity and/or expression does not align with their assigned sex at birth. Gender-affirming treatments have been shown to have a positive effect on the well-being of transgender individuals (1). Most individuals begin their transition at a young age. The medical transition for transgender adolescents (10-20 years old, based on WHO criteria) involves puberty suppression through the use of a GnRH agonist, which can be initiated early in puberty. Puberty blockers reduce behavioral and emotional problems and depressive symptoms, leading to improved daily functioning (2). Initiating puberty blockers is known as the "Dutch protocol" and is internationally applied in the transition of adolescents, proving to be successful in significantly reducing the severe distress caused by gender dysphoria (3).

Subsequently, from the age of 15, patients become eligible for cross-sex hormones, and from the age of 18, after at least 1 year of hormone therapy, they can undergo gender-affirming surgeries such as gonadectomy.

Hormonal and surgical steps in the transition can have reversible as well as irreversible effects on the fertility of transgender individuals. The advice of the World Professional Association for Transgender Health (WPATH) guideline is to counsel everyone about this, even if there is currently no desire for fertility preservation, for example, in adolescents(1). In the Knowledge and Care Center for Gender Dysphoria (KZcG), since 2021, we have established a protocol where all adolescents with gender dysphoria who start puberty suppression or cross-sex hormone therapy are counseled about the effects of treatment on their fertility and the possibilities for fertility preservation.

The number of adolescents with gender dysphoria seeking care at the Knowledge and Care Center for Gender Dysphoria (KZcG) has been increasing annually (Figure 1). As a result, we are seeing a larger group of adolescents in our clinic to discuss fertility preservation. Part of them assigned male at birth (i.e. transfeminine). The rise in number of adolescents is similar to other countries across the world, for example, in Australia(4).

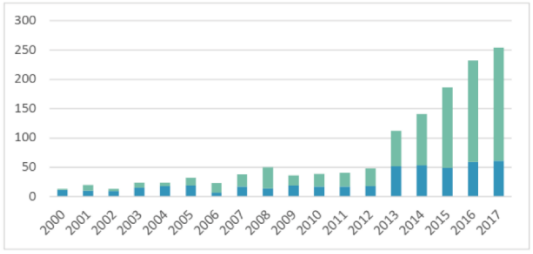

Figure 1: Number of registrations per year of children and adolescents at the KZcG

For this group, it is particularly important to provide fertility counseling before starting puberty blockers. Because, puberty

	blockers prevent or stop the production of testosterone, thereby inhibiting the development of secondary sexual characteristics. As a consequence, this also prevents or greatly reduces spermatogenesis, (i.e. the maturation of sperm cells). From Tanner stage 2, adolescents in our center become eligible for puberty suppression. However, spermatogenesis may not have started in everyone by that stage. It is often not possible to obtain mature sperm cells from ejaculation due to both the physical and mental inability to induce ejaculation, caused by dysphoria and age. Therefore, the option of cryopreserving ejaculated spermatozoa (semen cryopreservation) is often not available. For individuals unable to ejaculate, testicular sperm extraction (TESE) is offered. In this procedure, tissue is taken from the testes through biopsies, with the aim of finding mature sperm cells in the laboratory and freezing them for future use. The care for transgender adolescents was established in 2021 at Amsterdam UMC, location AMC. Since 2014, in the Netherlands, the moratorium that prohibited the use of surgically obtained sperm for achieving pregnancy, which had been in effect since 1996, was lifted after the publication of the results of the study titled "ICSI with testicular sperm in men with azoospermia: an observational study with follow-up of children" conducted by Radboud UMC and Amsterdam UMC (5, 6). These studies demonstrated the safety of testicular sperm extraction (TESE) by monitoring pregnancies and children born through ICSI with testicular sperm. Since then, quality standards have been established, and the TESE procedure has been recognized and deemed safe. It has since become a standard clinical practice in specialized centers and can be used, among other cases, for fertility preservation in men with (testicular) cancer, Klinefelter patients without current desire for children, or trans women with azoospermia.

	Furthermore, there is a significant amount of expertise in taking testicular biopsies in adolescents, particularly in the context of pre- and peripubertal oncology patients undergoing infertility-inducing treatments like chemotherapy. Since 2011, small pieces of testicular tissue have been frozen before the start of the treatment in these patients, allowing for the potential isolation and multiplication of stem cells from the frozen tissue in the future, to be reintroduced into the testes to fulfill the desire for children through natural conception (7). The operative risks associated with this procedure, such as infection and bleeding, are low, ranging from 1.3% to 2.6% (8). Additionally, no long-term negative effects have been observed in this group (9, 10). However, for transfeminine adolescents, this technique cannot be offered at present due to the need for testosterone replacement, which is suppressed in trans women through testosterone blockers and gender-affirming female hormones, or due to prior gonadectomy. Therefore, for the prepubertal execution of the TESE procedure, successful in vitro spermatogenesis must first be developed. To maximize the chances of a successful TESE, spermatogenesis should already be underway, for which the production of testosterone is crucial. However, testosterone is also responsible for (irreversible) virilization, such as deepening of the voice and facial hair growth. Therefore, it is highly burdensome for this group of patients to postpone puberty suppression in order to secure their fertility for the future, especially considering that active fertility desire does not exist at the start of puberty. Currently, there is a lack of valid clinical factors to predict when spermatogenesis has sufficiently progressed in transfeminine adolescents. The intratesticular testosterone level is significantly higher than the peripheral blood testosterone level, and the blood testosterone value is not determinative of the intratesticular level. Ideally, a high intratesticular testosterone level would be beneficial for

	sperm production, while maintaining the lowest possible peripheral testosterone level to minimize virilization. In the current clinical practice, the child endocrinologist determines the Tanner stage before initiating puberty suppression, followed by fertility counseling with the gynecologist. If fertility preservation is desired afterward, a referral to the andrologist is made to assess the indication for TESE and determine the optimal timing for the procedure. In cases where ejaculation is not possible, the andrologist uses ultrasound measurements of testicular volume, serum testosterone, LH, and FSH levels to make this determination. Recent Australian research in transfeminine adolescents has shown that testicular volume appears to be the most predictive factor. This finding is based on a retrospective cohort study with a small sample size of N=31 (11). If the TESE procedure is performed too early, it is possible that no or insufficient suitable sperm cells can be found. TESE is an invasive procedure that can have complications such as infection and bleeding, although the chances of these complications are low. However, complications may occur even without freezing sperm cells. Additionally, it should be noted that the patient is constantly reminded of their genitalia during the postoperative period, which can cause dysphoria. The procedure should be performed in a way that avoids permanent damage to the testes, ensuring that the process is reversible in case the patients choose their assigned gender at birth or if there is sufficient tissue available to assess the presence of sperm cells at the time of gonadectomy. Puberty progresses differently in each individual. In some cases, secondary changes occur faster than sperm maturation, leading to high levels of mental distress due to dysphoria, which may cause patients to refrain from undergoing TESE. This period can also be stressful and uncertain for both the child and the parents. Therefore, it

	is important to provide comprehensive counseling and multidisciplinary support throughout the process. Currently, there are no ideal clinical non-invasive parameters to determine the optimal timing for TESE. To determine the appropriate moment for a successful TESE there is need to set up a cohort study of all transfeminine adolescents who are referred for TESE. This will enable us to make better predictions in the future and optimize our fertility counseling and care. Furthermore, we intend to improve the counseling and support provided to both parents and the child by examining the experiences of the referred group throughout the entire process and reflecting on the experiences of the group that has undergone TESE.
Objective	When is the TESE process most successful by: 1. Identifying clinical non-invasive parameters that indicate a high likelihood of finding mature sperm cells. 2. Evaluating the experience of the process for patients and parents to determine the potential physical and emotional burden of the TESE process and identifying factors that can reduce this burden to improve feasibility.
Study design	For part 1 of the research question "when is the sperm yield from TESE most successful," the study design is observational, including both retrospective and prospective cohort research, utilizing data from medical records. To address the second part of the research question, we intend to employ a descriptive research method. This will involve qualitative research through interviews and quantitative research through questionnaires administered to both the children and their parents during or after the treatment.
Study population	Healthy adolescents assigned male at birth with gender dysphoria and a desire for surgical fertility preservation prior to or after the

	temporary discontinuation of puberty suppression, who have been referred to the andrologist
Inclusion criteria	Patients will be included if they meet the following criteria: • (Parents of) Individuals assigned male at birth who identify as transfeminine (trans girl, non-binary person, etc.). • Under the care of a specialized gender center or clinic • Have a diagnosis of gender dysphoria according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) or a diagnosis of gender incongruence according to the International Classification of Diseases, 11th Revision (ICD-11). • Have not yet started or have discontinued puberty suppression before the start of gender affirming hormone treatment. • Referred or scheduled for referral to the andrologist following fertility counseling.
Exclusion criteria	Patients will be excluded if they do not wish to participate in this research.
Sample size	We aim to conduct the study over a period of 2 years to include a sufficient number of 30-40 adolescents, allowing us to generate valid data.
Recruitment of participants	The participants who have already undergone TESE will be approached by the primary treating physician during their clinic visit or via telephone. The participant information will then be sent to them via email or mail. During the next physical appointment that the participant has at gender clinic, the consent form will be signed in the presence of the researcher. If the referral has not yet been made, the primary treating physician will inform the participant about the study during the fertility counseling session and provide them with the participant information after the consultation. If a referral for TESE is desired, the primary treating physician will contact the participants and their parents by phone to confirm their interest in participation. A one-week

	consideration period will then begin. If participation is desired, the consent form will be signed during the next physical appointment in the presence of the researcher. If the participant has a long distance to travel to the gender clinic and does not have any upcoming physical appointments in the near future, the consent form will be sent by mail with a prepaid return envelope.
Intervention	The participant information will include a request for permission to use data from the medical records for scientific research purposes. For the second part of the research question, we will also inquire whether the participant and their parents are willing to complete a questionnaire at least once and up to three times, with each questionnaire taking a maximum of 10 to 15 minutes. Additionally, a minimum of 5 study participants who have undergone TESE will be asked to participate in an interview, as well as their parents. Similarly, a minimum of 5 study participants who are referred to the andrologist will also be invited to participate in an interview, along with their parents. The interviews will take place once at location or via video call if there is a long travel distance, and they will last a maximum of 1 hour.
Standard care / Standard treatment	All adolescents are referred for fertility counseling before starting puberty suppressants. In this consultation, a counseling session takes place regarding fertility preservation. If the patient expresses a desire for preservation and is eligible for TESE (due to age/early puberty/incapacity to produce ejaculation), they are referred to the andrologist. An intake appointment is scheduled, after which the indication for TESE is confirmed. Currently, the indication is based on blood values (testosterone, LH, FSH) and an ultrasound to determine testicular volume. If the testicular volume or laboratory values are still insufficiently indicative of a successful TESE, the examination is repeated after 3 to 6 months or upon the patient's request if puberty progresses more rapidly and intensely than expected, thus tailored to the patient. The TESE procedure is performed in an outpatient setting under general anesthesia with local anesthesia, using microsurgery

	techniques to obtain small pieces of testicular tissue, ensuring reversibility of the procedure.
Study parameters	Primary outcome measures: Part 1: Ultrasound-determined testicular volumes before TESE procedure, when this is not available, we will use the measurements of the Tanner stage at the time of the procedure, by the pediatric endocrinologist. Laboratory values (testosterone, LH, FSH) shortest before the TESE procedure, including subsequent measurements, collected as part of routine clinical care for non-study purposes Age at the time of TESE Presence of spermatozoa Number of cryopreserved straws Quality of obtained sperm cells (concentration/motility) Part 2: Experience of the TESE procedure for the patient and parents Experience of counseling for the patient and parents Experience of any waiting period for the patient and parents Feasibility for adolescents: Outcome of TESE after referral (whether the TESE procedure was carried out or not)
Study endpoints	Secondary outcome measures: Progression of secondary sexual characteristics/virilization from the time of referral to the TESE procedure Subgroup of patients who temporarily discontinued puberty suppressants
Statistical analysis	The statistical program SPSS will be used for analyzing the observational cohort and questionnaires. Descriptive analyses will be conducted to display the central tendency (mean, median, and mode) and variation (range and standard deviation) of the included sample.

	To demonstrate a statistically significant relationship between different variables and the observed outcomes, linear and multivariate regression analyses will be employed. If necessary, adjustments will be made for confounding factors. The questionnaires will be send via Castor and analyzed using SPSS. The interviews will be transcribed verbatim. Transcripts will be coded using the MaxQDA software program, from which thematic analysis themes will be derived.
Burden for the participant	The interviews are conducted face-to-face or, in the case of long travel distances, through video consultations. The interviews last for a single session of 45-60 minutes. The questionnaires are sent online and take approximately 15 to 20 minutes to complete. The topics of fertility and the physical changes related to the TESE procedure discussed in the interviews can be considered intimate information. However, it is important to note that these topics are also routinely discussed by physicians and psychologists during clinical visits. Therefore, participants may find it relatively normal to discuss these topics with their healthcare providers. Nevertheless, participants have the option to select "I do not want to complete this questionnaire" for each questionnaire, in which case the questions will be removed from the digital survey. Apart from the time invested in completing the questionnaires and/or participating in interviews, there is no additional burden on the participants. Blood tests are always part of the treatment process and there are no additional blood sampling moments.
Risk for the participant	The participant faces no risks.
Benefits of participating in the research	The participant does not have direct benefits from participating in the research. Indirect benefits may include the potential improvement of care and information provision within the Knowledge and Care Center for Gender Dysphoria, both nationally and internationally, based on the research outcomes. This can be perceived as highly meaningful.

Disadvantages of participating in the research	The participant has no disadvantages of participating in the research.
Compensation for the participant	The participant will not receive any compensation for participating in the research.
Administrative aspects	The patient characteristics and treatment details (success, outcomes, etc.) will be collected from the medical records and stored in a pseudonymized SPSS file, which is password-protected. The database is stored on a secured departmental drive and is accessible only to the authorized researchers. Access to the database is monitored and recorded. The questionnaires will be administered using Castor EDC Surveys. This ensures that all data is stored in an encrypted format. The research data and the resulting database will be retained for up to 15 years after the completion of the study and will then be destroyed.

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