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BMJ Open TurnerFertility trial: PROTOCOL for an observational cohort study to describe the efficacy of ovarian tissue cryopreservation for fertility preservation in females with Turner syndrome

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ABSTRACT

Objective To investigate the occurrence of live birth in women with Turner syndrome (TS) after ovarian tissue cryopreservation in childhood followed by auto transplantation in adulthood and to find reliable prognostic markers for estimating the ovarian reserve in girls with TS in the future.

Setting An observational cohort study with long-term follow-up in a tertiary fertility clinic in the Netherlands. Patients recruitment between January 2018 and December 2021.

Participants 100 females aged 2 through 18 years with classical Turner (ie, 45,XO) or Turner variants (ie, 45,X mosaicism or structural anomalies). Girls with Y chromosomal content, minor X deletions with marginal impact on fertility, active HIV, hepatitis B or hepatitis C infection, and/or an absolute contra indication for surgery, anaesthesia or future pregnancy will be excluded.

Interventions Ovarian cortical tissue will be harvested by performing a unilateral oophorectomy via laparoscopic approach. Ovarian cortex fragments will be prepared and cryopreserved. One fragment per patient will be used to determine follicular density by conventional histology, and to perform fluorescence in situ hybridisation analysis of ovarian cells. Routine chromosome analysis will be performed on both lymphocytes and buccal cells. A blood sample will be taken for hormonal analysis and all subjects will undergo a transabdominal ultrasound to determine the uterine and ovarian size. Patient characteristics, pregnancy rates and pregnancy outcomes will be collected from the patient's medical record.

Ethics and dissemination The study protocol has been approved by the Central Committee on Research Involving Human Subjects in November 2017 (CCMO NL57738.000.16).

Trial registration number NCT03381300.

BACKGROUND

Turner syndrome (TS) is the most common chromosomal abnormality among females, affecting one in 2500 live born girls.^{1–3} Due to the partial or complete absence of one of

Strengths and limitations of this study

- Patients were involved in the initiation and design of this study protocol.
- The long-term follow-up of this study provides the unique opportunity to study the efficacy of ovarian tissue cryopreservation and pregnancy outcomes in females with Turner syndrome.
- The single-centre design could be a limitation.

the two X-chromosomes the fetal development is affected, leading to abnormalities in almost all organs including the gonads.⁴ Signs and symptoms vary among girls with TS, and intelligence is generally normal. The most common phenotypic features are infertility, short stature and cardio-aortic malformations.

Females with TS are known to have a limited reproductive lifespan due to an accelerated loss of germ cells. This process starts during the fetal period and continues until the point when the ovarian reserve is empty.^{5,6} Previous research has shown that primordial follicles can still be found in the ovaries of young girls with TS.⁷ However, in most females with TS the ovarian reserve is exhausted before reaching adulthood.⁶ Approximately one-third of females with TS have some pubertal development and 10%–15% will experience one or more spontaneous menstruation cycles.^{8–10} Spontaneous pregnancies occur in approximately 2.0%–7.6% of women with TS.^{11–15} Several interview studies^{16,17} show that, regardless of age, uncertainty about their fertility is one of the major concerns for girls and females with TS and their parents. While fertility preservation has garnered greater attention in the media, physicians are frequently asked whether these methods



could also be used to preserve the fertility of patients with TS.

Fertility preservation includes the cryopreservation of the patient's own gametes, either by preserving mature oocytes or ovarian tissue containing primordial follicles. Cryopreservation of mature oocytes (OC) is a proven fertility preservation approach but requires ovarian activity, a good ovarian reserve and psychological maturity.¹⁸ This method is limited to a small percentage of females with TS, namely those who will be fertile after a spontaneous onset of puberty and menstruation. Furthermore, the patient has to be emotionally mature enough to undergo the procedure, which involves ovarian stimulation with exogenous follicle stimulating hormone (FSH) administration followed by transvaginal ultrasound-guided oocyte retrieval.¹⁸

Because of the limitations of OC in girls with TS, ovarian tissue cryopreservation (OTC), appears to be a more promising technique for fertility preservation for this condition. The procedure can be performed in patients with TS regardless of their age or ovarian activity, and probably offer more females with TS the possibility to store a number of primordial follicles before their disappearance.¹⁹ OTC is a proven method to preserve the fertility of young females at risk of iatrogenic premature ovarian insufficiency such as females undergoing gonadotoxic cancer treatments.^{20–22} Auto transplantation of cryopreserved-thawed ovarian cortical tissue in cancer survivors has resulted in restoration of ovarian function in 67%–93% of cases^{23–26} with reported live birth rates (LBRs) between 25% and 33% per transplantation.^{24 25 27 28} A recent study with a limited number of patients even reported a LBR up to 75%.²⁶ Over the past decades, several clinical guidelines^{29–38} and decision tools^{39–44} for OTC have been developed for these patients.

From 2002, OTC procedures have also been performed experimentally in at least 83 young females with TS.^{7 19 45–47} In approximately one quarter of females with TS, follicles were present.¹⁹ Unfortunately, optimal discriminative markers for the presence or absence of follicles in females with TS are currently lacking. However, there is a general agreement that the mosaic karyotype is the most promising group to have ovarian follicles and to benefit from fertility preservation.^{18 19 48 49}

Furthermore, there are to date no published records of girls with TS who have returned for autotransplantation. As long as the efficacy of fertility preservation in females with TS regarding future pregnancy and live birth is unknown, experts might be reluctant to recommend routine OTC procedures in females with TS.^{18 19 50 51}

Exploring the efficacy of fertility preservation in females with TS seems a logical next step.⁴⁸ There is a strong need for a structured observational cohort study with long-term follow-up. This study should focus on the efficacy of OTC in females with TS including pregnancy rates and pregnancy outcomes, and on the development of a reliable prognostic model for estimating the ovarian reserve in females with TS. Ideally, a decision aid based

on the information needs of females with TS and their parents should be developed with the aim to help them to make a deliberate decision between OTC and the alternative options for future parenthood. This decision aid should include a reliable prognostic model to estimate the ovarian reserve in females with TS. Herewith, the urgency of fertility preservation could be determined and unnecessary surgical procedures can be avoided.

OBJECTIVE

To investigate the occurrence of live birth in women with TS after OTC in childhood followed by auto transplantation in adulthood and to find reliable prognostic markers for estimating the ovarian reserve in girls with TS in the future.

METHODS

Study design

An observational cohort study with long-term follow-up in a tertiary fertility clinic in the Netherlands. This study is registered in ClinicalTrials.gov. The study flow is visualised in figure 1.

Study period

Recruitment started January 2018. OTC procedures will be performed between January 2018 and December 2021. We expect to end this study in 2071.

Interventions

Based on the international Cincinnati Turner Guideline Consensus Meeting, July 2016⁵² and the consultation of Dutch cardiologists, paediatric cardiologists and anaesthesiologists between 2016 and 2017, there are no absolute cardiovascular contra-indications for surgical intervention and/or pregnancy. Advice against surgical intervention and/or pregnancy should be based on the patient-specific cardiovascular risk profile.

Therefore, all girls will undergo preoperative screening and risk assessment by a paediatric cardiologist and paediatric anaesthetist. After this screening, they will be defined as either low-risk participants or high-risk participants based on their individual risk profile. High-risk patients and their parents will be informed about their risks and excluded from this study in order to ensure patient safety. In low-risk participants the surgical procedure, followed by a hospital stay of one night, will be planned.

Ovarian cortical tissue will be harvested by performing a unilateral oophorectomy via laparoscopic approach. A collaborating team of well-trained paediatric surgeons, gynaecologists specialised in reproductive medicine, and laboratory workers specialised in human ovarian cortical tissue cryopreservation, will ensure safe and efficient cryopreservation of the ovarian tissue. Cryopreservation of the ovarian tissue fragments will be performed according to the Dutch protocol 'Cryopreservation and

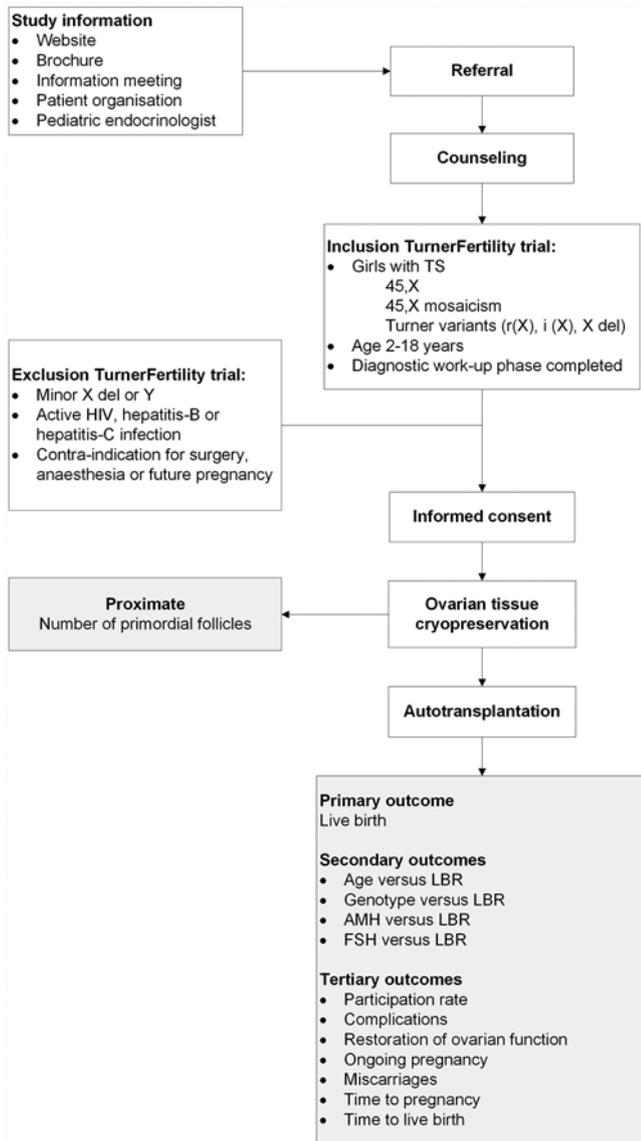


Figure 1 Flowchart of study. AMH, anti Müllerian hormone; FSH, follicle stimulating hormone; i(X), isochromosome X; LBR, live birth rate; r(X), ring chromosome X; TS, Turner syndrome; X del, X deletions; 45,X, 45,X Monosomy.

transplantation of ovarian tissue' (Dutch Network Fertility preservation, September 2012).

One cortex fragment per patient of approximately 8×5×1mm will be used to determine follicular density by conventional histology, and to perform fluorescence in situ hybridisation (FISH) analysis of three different ovarian cell types (oocytes, granulosa cells and stromal cells).⁵³ Routine chromosome analysis will be performed on both lymphocytes and buccal cells. If follicles are present, additional FISH analysis of urine cells will be performed. Furthermore, a blood sample of 3.5mL will be taken for hormonal analysis (ie, FSH, luteinizing hormone (LH), anti Müllerian hormone (AMH), oestradiol and inhibin B). All subjects will undergo a transabdominal ultrasound to determine the uterine and ovarian size. Information such as the patient's age and the spontaneous onset of puberty and menstruation will be collected

from the patient's medical record. In the future, orthotopic transplantation of the auto graft is performed via laparoscopic or laparotomic approach.^{21 54–56} Pregnancy rates and outcomes will be collected from the patient's medical record.

Study population

The study population includes 100 females with TS.

Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- ▶ Girls and young females with classic Turner (ie, 45,X monosomy) or Turner variants (eg, 45,X mosaicism, ring X, isochromosome X, X deletions).
- ▶ Aged 2 through 18 years.
- ▶ Who completed the diagnostic work up phase of TS, according to the international guidelines, including routine cardiac screening.
- ▶ Whose agreement to participate in this study has been signed by both the parents (girls 2–11 years old).
- ▶ Whose agreement to participate in this study has been signed by the patient and her parents (girls 12–15 years old).
- ▶ Whose agreement to participate in this study has been signed by the patient (girls 16–18 years old).

Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- ▶ Girls and young females with minor X deletions with marginal impact on fertility or Y chromosomal content.
- ▶ Girls with an active HIV, hepatitis B or hepatitis C infection.
- ▶ Girls with an absolute contra-indication for undergoing a laparoscopic unilateral oophorectomy under general anaesthesia and/or future pregnancy based on the patient specific risk profile (eg, severe cardiovascular co morbidity and/or body mass index (BMI) >40 kg/m²).

Recruitment of participants and informed consent

Girls with TS and their parents will be informed about this study by their paediatrician or by the Dutch TS patient organisation (Turner Contact Nederland (TCN)). Girls with TS who meet the inclusion criteria and are potentially interested, will receive the study information for patients and/or parents (<https://www.radboudumc.nl/trials/turner>) and will be invited for a general informative meeting. If the girls/parents are still interested after receiving this information, they will be referred to our hospital by their paediatric endocrinologist by using a specific form designed for this study. The personal counselling is done by a dedicated team of gynaecologists. A paediatric psychologist will be available for additional support. In case of comorbidity, a multidisciplinary team of gynaecologists, (paediatric)cardiologists and other

specialists if needed, will discuss the patient's specific risk profile.

Written consent must be given by the (parents of) patients before study participation. Girls and their parents are given time as individually requested to consider participation in this study. A pilot decision aid, website and age-specific information flyers have been developed, to help girls and their parents to make a deliberate decision (<https://www.radboudumc.nl/trials/turner>). The current decision aid includes a flowchart and background information on OTC in comparison with the existing options (ie, awaiting spontaneous pregnancy, vitrification of oocytes, oocyte donation, adoption and/or fostership). When a participant reaches the age of 16 years old, she will be asked for re-consent.

Biobanking

All biological material and data will be handled and stored according to the World Medical Association (WMA) Declaration of Taipei on ethical considerations regarding health databases and biobanks (67th WMA General Assembly, Taipei, Taiwan, October 2016). All patient data will be coded. Members of the research team, the Data and Safety Monitoring Board, and the Health Care Inspectorate are the only persons who have access to the key of the code. This key of code is stored in Castor Electronic Data Capture (EDC). The dignity, autonomy and privacy of the patients will be respected by the duty of confidentiality of all who are involved in handling data and biological material. There will be no discrimination.

EDC will be used for Good Clinical Practice (GCP)-compliant data collection. All participant data will be reported in electronic case report forms.

A safe storage of the ovarian tissue and consent forms is provided at our cryobank. This cryobank is ISO-accredited (accreditation number M101, ISO 15189), and registered by the Dutch Ministry of Health, Welfare and Sport (registration number 5515L/EO). The cryobank is located at a restricted area in the Radboud University Medical Centre, and access is permitted by electronic authorisation only.

All subjects will be asked for the continuation of the storage of the ovarian tissue yearly. Each individual patient and/or her parents may freely decide about the continuation of the storage of the ovarian tissue at our cryobank, transportation of the ovarian tissue to another cryobank, donation for research, or the elimination of their cryopreserved ovarian tissue. When the participant turns 16 years old, she will be asked for re-consent.

Subjects may withdraw from the trial at any time. Subjects do not need to state a reason for withdrawal.

Patient and public involvement

This study protocol has been initiated by and conducted with active input and feedback of international experts and patient representatives from the Dutch national patient organisation TCN and the differences of sex differentiation patient advisory group of the Radboud university medical centre. Patient representatives were

also involved in the development of patient information brochures, informed consent forms, and website. During the trial, patient representatives will be involved by the development of a decision aid and newsletters. Furthermore, surveys and focus groups among all counselled patients are held for continuous process improvement. All participants will be informed of the results of the TurnerFertility study through post or email.

International consensus to perform this study was achieved in a Delphi study including 35 medical professionals and 20 patient representatives from 16 different countries. This expert panel stated that OTC in patients with TS should be offered, but in a safe and controlled research setting only. The expert panel's standpoint was supported by eight key statements (Unpublished data).

OUTCOME MEASURES

Primary outcome

- ▶ Live birth after auto transplantation of cryopreserved-thawed ovarian cortical tissue (ie, LBR).

Proximate

- ▶ The number of primordial follicles found in the ovarian tissue.

Secondary outcomes

- ▶ The association between patient's age at cryopreservation and LBR.
- ▶ The association between patient's genotype and LBR.
- ▶ The association between patient's AMH level at cryopreservation and LBR.
- ▶ The association between patient's FSH level at cryopreservation and LBR.

Tertiary outcomes

- ▶ The study participation rate.
- ▶ The number of eligible participants.
- ▶ The age of the participant.
- ▶ The incidence of somatic mosaicism.
- ▶ The incidence of germ cell mosaicism.
- ▶ Serum hormone levels.
- ▶ The number of complications related to the laparoscopic procedure.
- ▶ The incidence of spontaneous puberty and/or spontaneous menarche after laparoscopic oophorectomy.
- ▶ The incidence of spontaneous pregnancies after laparoscopic oophorectomy.
- ▶ The incidence of menstruation cycle recovery after auto transplantation of cryopreserved-thawed ovarian tissue in the future.
- ▶ The incidence of pregnancies after auto transplantation of cryopreserved-thawed ovarian tissue in the future.
- ▶ The number of ongoing pregnancies after auto transplantation of cryopreserved-thawed ovarian tissue in the future.

- ▶ The number of miscarriages after auto transplantation of cryopreserved-thawed ovarian tissue in the future.
- ▶ The incidence of congenital anomalies in the offspring of women with TS who became pregnant after auto transplantation of cryopreserved-thawed ovarian tissue.
- ▶ Time to pregnancy after auto transplantation of cryopreserved-thawed ovarian tissue in the future.
- ▶ Time to live birth after auto transplantation of cryopreserved-thawed ovarian tissue in the future.

Data analysis

All data in this pilot study will be analysed on both intention-to-treat and per-protocol analysis. Data of girls who are lost to follow-up will be included as far as possible. Missing data will be reported along with the reason. Baseline data will be described quantitatively. Continuous variables will be summarised as means with SD or as medians with IQRs, depending on their distribution. Dichotomous and ordinal data will be summarised as percentages. For all analyses, IBM SPSS 25.0 will be used.

Descriptive statistics will be used to analyse the number of primordial follicles in the ovarian tissue, and the number of (ongoing) pregnancies, miscarriages and live born children after auto transplantation of cryopreserved-thawed ovarian tissue.

The primary outcome (dichotomous) 'live birth after auto transplantation of cryopreserved-thawed ovarian tissue' will be assessed and reported as a percentage. The number of primordial follicles (continuous) will be reported as a mean with SD. The number of pregnancies, ongoing pregnancies, miscarriages and congenital anomalies (dichotomous) will be reported as a percentage.

The time to pregnancy and the time to live birth after auto transplantation will be reported as a mean with SD.

Furthermore, the relationship between the age of the participant when OTC is performed (years), serum FSH level (IU/L), and serum AMH level (ng/mL), related to the number of primordial follicles, the incidence of (ongoing) pregnancies, live birth, and congenital anomalies will be described using Spearman's correlation coefficient. In addition, the number of primordial follicles, the incidence of (ongoing) pregnancies, live birth, and congenital anomalies will be described by the patient's karyotype (ie, 45,X monosomy or mosaicism).

Interim analyses

Interim analyses on safety and fertility are planned every 6 months until the last participant (n=100) has undergone the laparoscopic unilateral oophorectomy followed by OTC. The first interim analysis will be performed after the inclusion of the first 10 patients. Each interim analysis will be reported to the independent *Data and Safety Monitoring Board*. The percentage of participants with follicles in their ovary will be used as a fertility indicator. The percentage of participants who

had one or more complications related to the laparoscopic oophorectomy and/or anaesthesia will be used as a safety indicator.

Sample size calculation

The estimated LBR related to OTC in girls with TS is currently unknown. Hence, the sample size calculation should be based on LBR in other patient groups. The LBR per transplantation of earlier cryopreserved ovarian tissue in cancer survivors is approximately 25%.²¹ In order to describe the dichotomous outcome LBR, we used the sample size calculation of Hulley *et al.*⁵⁷ We would need a total sample size of 72 participants if the LBR in females with TS would be similar.

However, one should consider that auto transplantation will not be performed in all girls who are participating in this study, due to several reasons (eg, absence of follicles, future contra indications and/or on patient's preference). Furthermore, females with TS show a higher risk of miscarriage,¹³ and a slightly higher risk of a child with a congenital disorder when they conceive spontaneously.^{13 58} If these increased risks are related to the functional integrity or the chromosome profile of their follicular cells remains unclear. On the other hand, performing an exploratory intervention study in >100 minors, is unrealistic and inappropriate. Therefore, we aim to include primarily a total number of 100 girls with TS in this 'proof of concept' study.

DISCUSSION

This study is an example of patient-initiated research as there is an increasing demand for fertility preservation options in girls and young females with TS.⁴⁸ This study protocol was developed together with patient representatives and gives females with TS the possibility to undergo an experimental fertility preservation procedure in a safe and controlled research setting. The long-term follow-up of this study up to live birth provides the unique opportunity to study the efficacy of OTC and pregnancy outcomes in females with TS. Furthermore, this study could contribute to the development of a reliable prognostic model for estimating the ovarian reserve in females with TS in the future.

A limitation of this study could be the single-centre design combined with the relatively low expected LBR. For the development of a prediction model, more patients should be included in the analysis to increase the internal validation. Furthermore, an external data validation should be performed. We suggest an international, multicentre study with a clear study protocol and a central registration after the results of the interim analysis of this pilot study have been published and are open for collaborating with other experts within the field of fertility preservation in females with TS.

ETHICS AND DISSEMINATION

This study protocol was conducted with input and feedback of patient representatives and international experts.

Ethical approval by the Dutch Central Committee on Research Involving Human Subjects was obtained in 2017 (CCMO NL57738.000.16) and is in accordance with the Declaration of Helsinki, the Medical Research Involving Human Subjects Act (WMO), the Guideline for GCP, and all other applicable regulatory requirements. An independent Data and Safety Monitoring Board has been established to perform interim analyses on safety and futility. Results will be disseminated through peer-reviewed publications and presentations at international scientific meetings.

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Contributors KF, JvdV, RP, DB, IB, RvG and MS have designed the trial and are accountable for all aspects of the work. MS is responsible for the overall logistical aspects of the trial, the data collection and analysis, and the draft of this paper. KF, JvdV, DB, IB, RvG and RP revised the draft critically and approved the final version of the study protocol. KF, JvdV, DB, IB and MS included patients, KF is performing the surgical procedures, and RP and MS are responsible for the laboratory work.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Approved by the Dutch Central Committee on Research Involving Human Subjects (2017).

Provenance and peer review Not commissioned; externally peer reviewed.

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