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TurnerFertility trial: fertility preservation in young girls with Turner syndrome by freezing ovarian cortex tissue—a prospective intervention study

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Objective: To evaluate which girls with Turner syndrome (TS) could benefit from fertility preservation by ovarian tissue cryopreservation on the basis of karyotype, puberty status, and hormonal data.
Design: Prospective intervention study; participants were included between 2018 and 2020.
Setting: Tertiary hospital in the Netherlands.
Patients: In total, 106 girls with TS aged between 2 and 18 years were included. Girls with minor X chromosome deletions, Y chromosomal content, active infections, or contraindications for surgery were excluded.
Intervention: A laparoscopic unilateral ovariectomy was performed to obtain ovarian cortical tissue for cryopreservation. One tissue fragment per participant was used to determine the number of follicles per ovary by serial sectioning and staining. Chromosome analysis was performed on lymphocytes and buccal cells. A blood sample was taken before the ovariectomy for hormonal analysis.
Main Outcome Measures: The presence of follicles in ovarian cortex tissue from girls with TS in relation to karyotype, puberty status, and hormonal data.
Results: A unilateral ovariectomy was performed on 93 girls with TS. Complications after surgery occurred in 5 girls, including luxation of psychological symptoms in 2 girls. In 13 (14%) girls, a 46,XX cell line was found in buccal cells that was absent in lymphocytes. Follicles were observed in 30 (32%) of the 93 girls and were found mainly in girls with a 46,XX cell line in lymphocytes or buccal cells (Phi coefficient = 0.55). Spontaneous onset of puberty (Phi coefficient = 0.59), antimüllerian hormone (AMH; point-biserial correlation

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Data (karyotype lymphocytes/buccal cells, puberty status and/or hormone levels) regarding 24 subjects participating in the TurnerFertility trial has previously been published:

- Peek et al., Ovarian follicles of young patients with Turner's syndrome contain normal oocytes but monosomic 45,X granulosa cells. Hum Reprod. 2019
- Nadesapillai et al., Why are some patients with 45,X Turner syndrome fertile? A young girl with classical 45,X Turner syndrome and a cryptic mosaicism in the ovary. Fertil Steril. 2021
- Schleedoorn et al., Why Turner patients with 45, X monosomy should not be excluded from fertility preservation services. Reprod Biol Endocrinol. 2022
- Peek et al., Assessment of folliculogenesis in ovarian tissue from young patients with Turner syndrome using a murine xenograft model. Fertil Steril. 2023

Pseudonymized patient data, study protocol, analysis plan, or other specific data sets can be made available after request. Proposals should be directed to the corresponding author S.N.
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[r] = 0.82), inhibin B (r = 0.67), and follicle-stimulating hormone (r = −0.46) levels were also correlated strongly with the presence of follicles. Furthermore, AMH levels had a significant correlation with the number of follicles per ovary (r = 0.66).
Conclusion: Favorable predictive markers for the presence of follicles included either a 46,XX cell line, spontaneous onset of puberty, or a combination of measurable AMH and normal follicle-stimulating hormone levels. Karyotyping of two peripheral cell lines in girls with TS is recommended to reveal hidden mosaicisms. Ovarian tissue cryopreservation should be offered with caution in a research setting to those with a sufficient ovarian reserve, considering the significant loss of follicles after ovarian tissue cryopreservation and autotransplantation. Physicians should pay attention to the mental health of the girls during the whole process.
Clinical Trial Registration Number: Trial registration number: NCT03381300- Preservation of Ovarian Cortex Tissue in Girls With Turner Syndrome - Full Text View - ClinicalTrials.gov. Registered on: December 21, 2017. First patient recruited on January 1, 2018. (Fertil Steril® 2023;120:1048–60. ©2023 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Turner syndrome, ovarian tissue cryopreservation, fertility preservation, premature ovarian failure

Turner syndrome (TS) is a genetic condition that is caused by partial or complete absence of one sex chromosome (1). This may affect multiple organ systems, resulting in cardiac abnormalities, short stature, and premature ovarian failure (2, 3).

Signs of a depleted ovarian reserve occur in the majority of girls with TS at an early age (4–6). Only 30% of girls with TS will have spontaneous breast development, and 10%–15% will experience spontaneous menarche (4, 7, 8). Spontaneous pregnancies are rare and only occur in approximately 2%–14% of cases (8, 9). In addition, there is also a high risk of miscarriage (31%–48%) (8, 10).

Both girls and women with TS report infertility as an important concern (11, 12). Unfortunately, the options to preserve fertility in girls with TS are limited (11, 13). Cryopreservation of mature oocytes is the only approved fertility preservation treatment for girls with TS, but this treatment is restricted mainly to those who have ovarian activity after puberty (13–15). Most women with TS therefore depend on alternative options (such as foster care, adoption, or oocyte donation) to become parents (11).

Ovarian tissue cryopreservation (OTC) could be an option for girls with TS to preserve primordial follicles before premature ovarian failure occurs (13). OTC has already been performed in an experimental setting by several research groups in girls with TS (14, 16–19). These studies demonstrated the presence of follicles in the ovarian cortex of mainly mosaic girls with TS, but it remains unclear which girls with TS are likely to benefit from OTC (20). Furthermore, there are still no pregnancy outcomes reported in girls with TS after using frozen-thawed ovarian tissue (21). For these reasons, the TurnerFertility trial was initiated to determine the live birth rate (LBR) in women with TS who underwent OTC during childhood and used cryopreserved ovarian tissue in adulthood. In this article, we described the first results of our study, focusing on the presence of follicles in ovarian cortex tissue in correlation with karyotype, puberty status, and hormonal parameters.

MATERIALS AND METHODS

Study design

The TurnerFertility trial is a prospective patient-initiated intervention study with long-term follow-up and is conducted at a tertiary clinic in the Netherlands. The main outcome of the trial is the LBR after OTC in women with TS. In the current article, the primary outcome is the presence of follicles in ovarian cortex tissue. The trial protocol has been published previously and was approved by the Dutch Central Committee on Research Involving Human Subjects (NL57738.000.16) (22). Approval was given to include between 100 and 110 girls with TS.

Patient selection

All girls with TS aged between 2 and 18 years in the Netherlands were eligible to participate after having completed the diagnostic workup for TS, including cardiac screening. Girls with minor X deletions with marginal impact on fertility, Y chromosomal content, active human immunodeficiency virus or hepatitis-B and C infections, or an absolute contraindication for surgery or future pregnancy (e.g., severe cardiovascular comorbidity or mental retardation) were excluded.

Girls with TS and their parents were informed about the trial by their pediatricians or by the Dutch TS organization. Those who were interested in the study were invited to an informative meeting, and thereafter referred to the tertiary clinic for individual fertility preservation counseling (23). During this consultation, girls with TS and their parents were informed about OTC, the risks of surgery, success rates of OTC in other patient groups, insights gained from already performed surgeries in girls with TS, the risks of pregnancy, and alternative options to fulfill future motherhood (11, 23). Age-adapted information and images were provided to inform young girls with TS about this study to promote their autonomy as much as possible. An appointment with a pediatric psychologist was offered for additional support during the trial. All included participants underwent preoperative screening with a pediatric anesthesiologist and pediatric surgeon.

Intervention

A transabdominal ultrasound was performed during counseling to examine the uterus and ovaries. During laparoscopic unilateral ovariectomy, performed by a pediatric surgeon and gynecologist, the largest ovary was removed to increase the likelihood of finding follicles in the ovarian cortex tissue. The ovary was transferred to cold Leibovitz-15 medium and directly transported to the laboratory. Cryopreservation of ovarian cortex tissue was performed as previously described

(24). Within eight weeks, participants and parents were contacted by phone and informed about the results and the number of cryopreserved tissue fragments. Furthermore, the occurrence of late adverse events and perceived care during the trial were evaluated.

Participants gave permission to use one cortex fragment of approximately $5 \times 8 \times 1.5$ mm for research purposes. One part of this cortex fragment was used to determine the follicular density by serially sectioning tissue at 4 μm thickness and staining with hematoxylin-eosin sections at 24 μm intervals. The remaining part of the fragment was saved for future research purposes.

Follicle density was determined as previously described (25). However, as the size of the ovaries varied widely between girls with TS, the estimated number of follicles in the whole ovary (follicle density multiplied by the weight of the cortex fragment and number of fragments) is a more accurate way to determine the ovarian reserve.

Buccal swabs were taken to examine an additional cell line for X chromosomal aberrations with fluorescence in situ hybridization (FISH). Karyotyping of 30 lymphocytes and FISH analysis of 100 buccal cells were performed (26). A blood sample was taken after general anesthesia for hormone analysis. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) concentrations were measured using electroluminescence immunoassay on a random-access analyzer (E801 Cobas, Roche; imprecision of 1%–4%). Inhibin B level was measured using an enzyme-linked immune assay (Inhib B gen II, Beckman Coulter; imprecision of 6%). The antimüllerian hormone (AMH) level was measured using a chemiluminescence immune assay on a random-access analyzer (Access, Beckman Coulter; imprecision of 3%). Cutoff points for AMH, LH, E_2, and inhibin B levels were chosen on the basis of whether the value was measurable. The cutoff point of 10 E/L for FSH level was chosen on the basis of literature and clinical experiences (27, 28).

Statistical analysis

The sample size was on the basis of the LBR per transplantation of cryopreserved ovarian tissue in childhood cancer survivors, considering that the ovarian reserve is lower and the miscarriage rate is higher in girls with TS, and that not every girl will be eligible for autotransplantation (22).

Descriptive statistics were used to describe baseline characteristics expressed as means with standard deviation or medians with interquartile range (IQR), on the basis of their distribution (Kolmogorov–Smirnov test). Ordinal and dichotomous outcomes were reported as percentages.

The correlation between the presence of follicles and the continuous variables age and hormone levels was determined using point-biserial correlation (r) (after log transformation of the hormones). The Phi coefficient (ϕ) was used to determine the correlation between the presence of follicles and karyotype, puberty status, and visibility of the ovaries. Pearson's correlation was used to determine the correlation between AMH levels and the number of follicles per ovary. Coefficients can range from -1 to $+1$ for negative or positive correlations. A coefficient of 0 indicates no association between variables.

Girls with TS were divided into four groups according to international definitions to analyze the correlation between pubertal stage and the presence of follicles: prepubertal girls (defined as aged <12 years with no signs of puberty), spontaneous thelarche (defined as Tanner stage 2), spontaneous menarche (defined as having at least one menstruation), and no puberty (defined as girls aged ≥ 12 years with no signs of spontaneous puberty). Statistical significance was set at $P \leq .05$. Data analysis was conducted using SPSS for Windows, version 27 (IBM Corp).

RESULTS

Participants

In total, 189 families attended the informative meeting. Subsequently, 173 (92%) of the 189 families opted for individual counseling. Informed consent was obtained from 106 (61%) of the 173 girls with TS and/or parents. Thirteen girls with TS dropped out of the study because of the low odds of having follicles or because they chose oocyte vitrification. Finally, 93 (88%) of the 106 girls underwent a unilateral ovariectomy.

Participant characteristics are shown in Table 1. The mean age at surgery was 11.9 years (range 3–19 years). Comorbidities were reported in 59 (63%) of the 93 girls. In 25 (27%) of the 93 girls, cardiac anomalies were present, of which the bicuspid aortic valve was the most common.

Ultrasound and surgery outcomes

In girls aged <12 years ($n = 43$), both ovaries were visible with abdominal ultrasound in 11 (26%) of the 43 girls; one ovary was visible in 5 (12%) of the 43 girls; and no ovaries were visible in 25 (58%) of the 43 girls. In two cases, no ultrasound was performed.

In girls aged ≥ 12 years ($n = 50$), both ovaries were visible in 17 (34%) of the 50 girls, one ovary was visible in 8 (16%) of the 50 girls, and no ovaries were visible in 27 (48%) of the 50 girls. One girl did not undergo an abdominal ultrasound examination. No anomalies of the uterus or ovaries were found. A moderate correlation was found between the visibility of the ovaries and the presence of follicles (Table 2).

In 33 (35%) of the 93 girls, a macroscopically normal ovary was visible during surgery. Follicles were found in 30 (32%) of the 93 girls. Complications after surgery occurred in 5 (5%) of the 93 participants. Two girls had a small skin hematoma after blood collection. One girl had a luxation of her latent esotropia and needed additional eye surgery. Another girl experienced feelings of sadness and insecurity after hearing that no follicles had been found in her ovarian tissue. The pediatric psychologist concluded it was a normal response given the situation and her age. Another girl developed suicidal thoughts after hearing that no follicles were present. A multidisciplinary team, including a psychiatrist, was able to stabilize her situation.

Karyotype

Karyotyping of both lymphocytes and buccal cells was performed in 92 (99%) of the 93 girls. In 13 (14%) of the 92 girls with 45,X in lymphocytes, an additional 46,XX cell line was

TABLE 1

Characteristics of participants sorted by karyotype.

Characteristics	All karyotype With follicles	All karyotype No follicles	45,X With follicles	45,X No follicles	46,XX present With follicles	46,XX present No follicles	Other[a] With follicles	Other[b] No follicles
N	30	63	1	27	22	11	7	25
Age at surgery, mean ± SD in y	11.8 ± 4.0	11.9 ± 4.4	14.7	11.0 ± 4.8	11.7 ± 4.2	12.6 ± 4.0	11.6 ± 4.0	12.6 ± 4.1
Comorbidities, n (%)[c]	15 (50.0%)	44 (70%)	1 (100%)	20 (74%)	11 (50%)	9 (82%)	3 (43%)	15 (60%)
Cardiac comorbidities, n (%)	10 (33%)	15 (24%)	1 (100%)	12 (44%)	7 (32%)	2 (18%)	2 (29%)	1 (4.0%)
Bicuspid aortic valve, n (%)	6 (20%)	10 (16%)	0	8 (30%)	4 (18%)	1 (9.1%)	2 (29%)	1 (4.0%)
Coarctation of the aorta, n (%)	1 (3.3%)	2 (3.2%)	0	2 (7.4%)	1 (4.5%)	0	0	0
Aortic dilatation, n (%)	3 (10%)	1 (1.6%)	1 (100%)	1 (3.7%)	2 (9.1%)	0	0	0
Other cardiac comorbidities	1 (3.3%)	4 (6.3%)	0	3 (11%)	1 (4.5%)	1 (9.1%)	0	0
Pubertal status								
Prepubertal, n (%)	12 (40%)	29 (46%)	0	14 (52%)	9 (41%)	4 (36%)	3 (43%)	11 (44%)
Spontaneous thelarche, n (%)	16 (53%)	9 (14%)	0	1 (3.7%)	12 (55%)	3 (27%)	4 (57%)	5 (20%)
Spontaneous menarche, n (%)	7 (23%)	1 (1.6%)	0	0	7 (32%)	1 (9.1%)	0	0
HRT, n (%)	3 (10%)	27 (43%)	1 (100%)	13 (48%)	1 (4.5%)	4 (36%)	1 (14%)	10 (40%)
Puberty induction, n (%)	1 (3.3%)	18 (29%)	1 (100%)	9 (33%)	0	3 (27%)	0	6 (24%)
HRT after spontaneous onset of puberty, n (%)	2 (6.7%)	9 (14%)	0	4 (15%)	1 (4.5%)	1 (9.1%)	1	4 (16%)
Other hormone therapy (GnRHa), n (%)	1 (3.3%)	0	0	0	0	0	1 (14%)	0
Hormone levels prior to surgery[d]								
N	26	36	0	14	21	7	5	15
FSH in E/L, median (IQR)	5.2 (2.2–7.7)	14 (4.4–53)	—	19 (5.0–59)	4.9 (2.2–7.8)	11 (5.6–60)	6.1 (2.6–8.1)	14 (2.9–40)
AMH in μg/L, median (IQR)	1.3 (0.45–2.8)	Below detection limit	—	Below detection limit	1.5 (0.8–3)	0 (0–0.3)	0.4 (0.1-1)	Below detection limit
LH in E/L, median (IQR)	1.0 (0–5)	0.8 (0–2.8)	—	0.84 (0–2.5)	1.0 (0–5.4)	1.2 (0.3–1.7)	0.9 (0.2–7.9)	0.4 (0–11)
Inhibin B in ng/L, median (IQR)	15.4 (0–35)	Below detection limit	—	Below detection limit	15 (0–37)	0 (0–3)	18.2 (7.0–47)	Below detection limit
Estradiol in pmol/L, median (IQR)	17.5 (0–115)	Below detection limit	—	Below detection limit	0 (0–105)	0 (0–150)	54.0 (0–175)	Below detection limit

Note: AMH = antimüllerian hormone; FSH = follicle-stimulating hormone; GnRHa = gonadotropin-releasing hormone agonist; HRT = hormone replacement therapy; IQR = interquartile range; LH = luteinizing hormone; SD = standard deviation.

[a] Other with follicles: 45,X/47,XXX (n = 1), isochromosome (n = 1), X chromosome deletion (n = 1), ring chromosome (n = 2), deletion of one X chromosome and additional part of the other X chromosome (n = 1), pseudoisodicentric X chromosome (n = 1).

[b] Other no follicles: isochromosome (n = 10), X chromosome deletion (n = 4), ring chromosome (n = 2), pseudoisodicentric X chromosome (n = 1), derivative X chromosome (n = 3), isodicentric X chromosome (n = 5)

[c] Comorbidities included: celiac disease, attention deficit hyperactivity disorder, thyroid disease, and horseshoe kidneys.

[d] Results of girls without hormone therapy.

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TABLE 2

Association between the presence of follicles and markers for the presence of follicles (age, karyotype, spontaneous puberty, visibility of the ovaries, and hormone levels).

Markers for the presence of follicles	Presence of follicles (n/total, %)	Correlation coefficient		
		All girls (93)	***Girls < 12 y (43)***	***Girls ≥ 12 y (50)***
Age[a]	All girls 30/93 (32%) Age ≥12 y 18/50 (36%) Age <12 y 12/43 (28%)	r = −0.01, *P*= .894 (n = 93)	r = −0.14, *P*= .360 (n = 43)	r = −0.18, *P*= .216 (n = 50)
Karyotype[b]	All girls 30/93 (32%) No 46,XX present 8/60 (13%) 46,XX present 22/33 (67%)	ϕ = 0.55, *P*< .001 (n = 93)	ϕ = 0.52, *P*< .001 (n = 43)	ϕ= 0.57, *P*< .001 (n = 50)
Spontaneous puberty[b] *(Prepubertal excluded)*	All girls 18/52 (23%) No 2/27 (7%) Yes 16/25 (64%)	ϕ = 0.59, *P*< .001 (n = 52)	ϕ = Not applicable (n = 4)	ϕ= 0.64, *P*< .001 (n = 48)
Visibility ovaries with ultrasound[b]	All girls 29/90 (32%) No ovary 8/49 (16%) One or both 21/41 (51%)	ϕ = 0.37, *P*< .001 (n = 90)	ϕ = 0.42, *P* = .007 (n = 41)	ϕ = 0.32, *P*= .024 (n = 49)
FSH[a] *(Girls without HRT)*	All girls 26/62 (42%) FSH >10 E/L 1/21 (5%) FSH ≤0 E/L 25/41 (61%)	r = −0.46, *P*< .001 (n = 62)	r = −0.56, P< .001 (n = 41)	r = −0.70, *P*< .001 (n = 21)
AMH[a] *(Girls without HRT)*	All girls 26/62 (42%) AMH <0.1 μg/L 1/34 (3%) AMH ≥0.1 μg/L 25/28 (89%)	r = 0.82, *P*< .001 (n = 62)	r = .89, *P*< .001 (n = 41)	r = 0.66, *P*= .001 (n = 21)
LH[a] *(Girls without HRT)*	All girls 26/62 (42%) LH ≥0.3 E/L 15/40 (38%) LH <0.3 E/L 11/22 (50%)	r = −0.02, *P*= .888 (n = 62)	r = −0.36, *P*= .021 (n = 41)	r = −0.12, *P*= .599 (n= 21)
Inhibin B[a] *(Girls without HRT)*	All girls 26/62 (42%) Inhibin B <10 ng/L 8/42 (19%) Inhibin B ≥10 ng/L 18/20 (90%)	r = 0.67, *P*< .001 (n = 62)	r = 0.62, *P*< .001 (n = 41)	r = 0.63, *P*= .002 (n = 21)
Estradiol[a] *(Girls without HRT)*	All girls 26/62 (42%) Estradiol <18 pmol/L 13/43 (30%) Estradiol ≥18 pmol/L 13/19 (68%)	r = 0.38, *P*= .002 (n = 62)	r = 0.13, *P*= .434 (n = 41)	r = 0.24, *P*= .292 (n = 21)

Note: AMH = antimüllerian hormone; FSH = follicle-stimulating hormone; HRT = hormone replacement therapy; LH = luteinizing hormone.
[a] The point-biserial correlation coefficient (r) is determined with the use of the continuous variable of the potential marker. Hormone levels were log transformed in case of a nonnormal distribution.
[b] The Phi (ϕ) coefficient is determined for the dichotomous potential markers.

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found in buccal cells. In 3 (23%) of these 13 girls, follicles were found. The girl, whose karyotyping of buccal cells was inadvertently not performed, had a 45,X/46,X,r(X) karyotype in lymphocytes. She was assigned to the "other" group given the very low probability of having a 46,XX cell line.

In 28 (30%) of the 93 girls with a 45,X karyotype in both lymphocytes and buccal cells, only one girl had follicles. In 33 (35%) of 93 girls with a 46,XX cell line in either lymphocytes and/or buccal cells, 22 (67%) of 33 girls had follicles. In 32 (34%) of the 93 girls with a structural aberration or 45,X/47,XXX karyotype, 7 (22%) of the 32 girls had follicles. The presence of a 46,XX cell line had a strong and significant positive correlation with the presence of follicles (Table 2).

Puberty

In our cohort, 41 (44%) of the 93 girls were at the prepubertal stage, and follicles were found in 12 (29%) of those 41 prepubertal girls. No significant correlation was found between the age of the girls and the presence of follicles (Table 2).

Spontaneous thelarche occurred in 25 (27%) of the 93 girls with TS, mainly in girls who also had a 46,XX cell line. Follicles were found in 16 (64%) of these 25 girls. Spontaneous menarche only occurred in 8 (9%) of the 93 girls, and in 7 (88%) of these girls, follicles were found. These girls all had a 46,XX cell line present. In girls aged $\geq$12 years without spontaneous puberty, only 2 (7%) of these 27 girls had follicles. Spontaneous puberty had a strong and significant correlation with the presence of follicles (Table 2).

In total, 30 (32%) of the 93 girls received hormone replacement therapy (HRT). Nineteen girls received estrogen monotherapy to induce puberty, and 11 girls received a combination of estrogen and progesterone. One girl received a gonadotropin-releasing hormone agonist (GnRHa). This girl had a structural chromosomal aberration and spontaneous thelarche at the age of 9 years. To increase the final height, the pediatrician started with GnRHa to inhibit puberty. Follicles were found in one girl with a 45,X karyotype using estrogen, in one girl with a 46,XX cell line using combination therapy, in one girl with a structural aberration using combination therapy, and in one girl with a structural aberration using GnRHa.

Hormone levels

Data of girls without HRT or GnRHa ($n = 62$) were used to analyze the association between different hormone levels and the presence of follicles (Table 2). This group included 41 prepubertal girls, 20 pubertal girls, and one girl without spontaneous puberty. In this group, 26 (42%) of the 62 girls had follicles. An overview of the hormone levels is shown in Supplemental Figure 1 (available online).

FSH levels (>10 E/L) were found in 21 (34%) of the 62 girls, of whom 7 were aged <12 years. FSH levels had a strong and significant negative correlation with the presence of follicles.

Antimüllerian hormone levels ($\geq$0.1 μg/L) were observed in 28 (45%) of the 62 girls, of whom 12 were aged <12 years. Antimüllerian hormone levels showed the strongest correlation of all markers with the presence of follicles, especially in girls aged <12 years. FSH levels ($\leq$10 E/L) combined with AMH levels ($\geq$.1 μg/L) were observed in 25 (96%) of the 26 nonHRT girls with follicles. In the group of girls that used HRT or GnRHa before surgery, only the girl with 45,X had an AMH level ($\geq$0.1 μg/L).

LH levels ($\geq$0.3 E/L) were observed in 40 (65%) of the 62 girls, of whom 20 were aged <12 years. The LH level was the only hormone without a significant correlation with the presence of follicles.

Estradiol levels ($\geq$18 pmol/L) were observed in 19 (31%) of the 62 girls, of whom 2 were aged <12 years. Estradiol only had a moderate and significant correlation with the presence of follicles in the whole group of girls with TS.

Inhibin B ($\geq$10 ng/L) was found in 20 (32%) of the 62 girls, of whom 8 were aged <12 years. Inhibin B had a strong and significant correlation with the presence of follicles.

Size of the ovary and estimated number of follicles

The size of the ovaries and the follicle density varied widely. In one girl, both a morphologically normal and a streaked ovary were present. In general, larger ovaries were observed in girls with a 46,XX cell line (mean 5,407 mm^3; range 500–24,000 mm^3) compared with girls without a 46,XX cell line (mean 2,276 mm^3; range 120–9,000 mm^3) and in girls $\geq$ 12 years (mean 2,797 mm^3; range 30–24,000 mm^3) compared with girls <12 years (mean 596 mm^3; range 30–3,888 mm^3). In all karyotype groups, pubertal girls with follicles had larger ovaries compared with prepubertal girls with follicles (mean 7,360 vs. 1,186 mm^3).

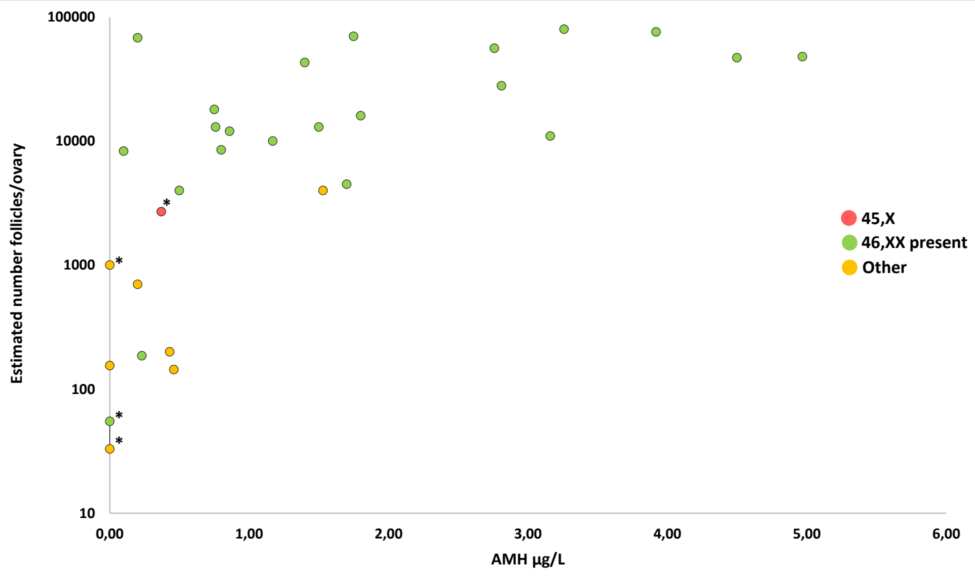
Girls with a 46,XX cell line had a greater calculated number of follicles per ovary (range 55–80,000 follicles) compared to girls with other karyotypes (range 33–4,000 follicles). In this cohort, AMH levels (<1 μg/L) mainly corresponded with a low number of follicles per ovary (Fig. 1). A significant correlation was found between AMH levels and the number of follicles per ovary ($r = 0.66$, $P<.001$).

Where many girls with TS only had primordial follicles in their ovarian cortex, some girls also had secondary or even antral follicles (Supplemental Fig. 2, available online). Abnormal primordial follicles were mostly seen in girls with a structural aberration (Supplemental Fig. 2I, available online).

Overview

Our results show different odds for the presence of follicles in different karyotypes. In Figure 2, an overview was created of puberty status, hormone levels, and the presence of follicles on the basis of the karyotype. In girls with a 46,XX cell line in lymphocytes or buccal cells (Fig. 2B), follicles were found more often. Both prepubertal girls and girls with spontaneous puberty (not using HRT) were more likely to have follicles when they had FSH levels ($\leq$10 E/L) and AMH levels ($\geq$0.1 μg/L).

FIGURE 1



Estimated number of follicles per ovary and antimüllerian hormone (AMH) levels in girls with Turner syndrome. The estimated number of follicles per ovary and the level of AMH in girls with follicles are presented (n = 30). In most girls, AMH levels (<1 μg/L) corresponded with a low number of follicles per ovary. However, exceptions were seen in which a high number of follicles per ovary was observed despite a low AMH level, and vice versa. The red dot represents the girl with 45,X. Green dots represent girls with a 46,XX cell line present in blood or lymphocytes, and orange dots represent girls with other karyotypes (e.g., structural aberrations and 45,X/47,XXX). *, girls receiving hormone replacement therapy or gonadotropin-releasing hormone agonist.

Nadesapillai. OTC in young girls with TS. Fertil Steril 2023.

DISCUSSION

In this article, the first results of the largest prospective study investigating the efficacy of OTC in girls with TS with long-term follow-up are presented. Follicles were present in 32% of the girls. We have observed that girls with TS and either a 46,XX cell line, spontaneous onset of puberty, or a combination of measurable AMH and normal FSH levels were most likely to have follicles in their ovarian cortex tissue.

Previous research groups have performed OTC on approximately 100 girls with TS (14, 16–19, 21). In these studies, girls with TS presented a variety of genotypic and phenotypic features. Karyotyping was often performed only in lymphocytes, but a clear description of the karyotyping method or the number of cells that were analyzed was generally not presented. In our study, chromosome analysis was performed on both lymphocytes and buccal cells to determine whether an additional cell line was present (26). An additional 46,XX cell line was present in 13 girls, of whom 3 had follicles. These findings show that screening of a single cell line could lead to unfairly categorizing girls in the 45,X karyotype group with an unfavorable fertility prognosis (29).

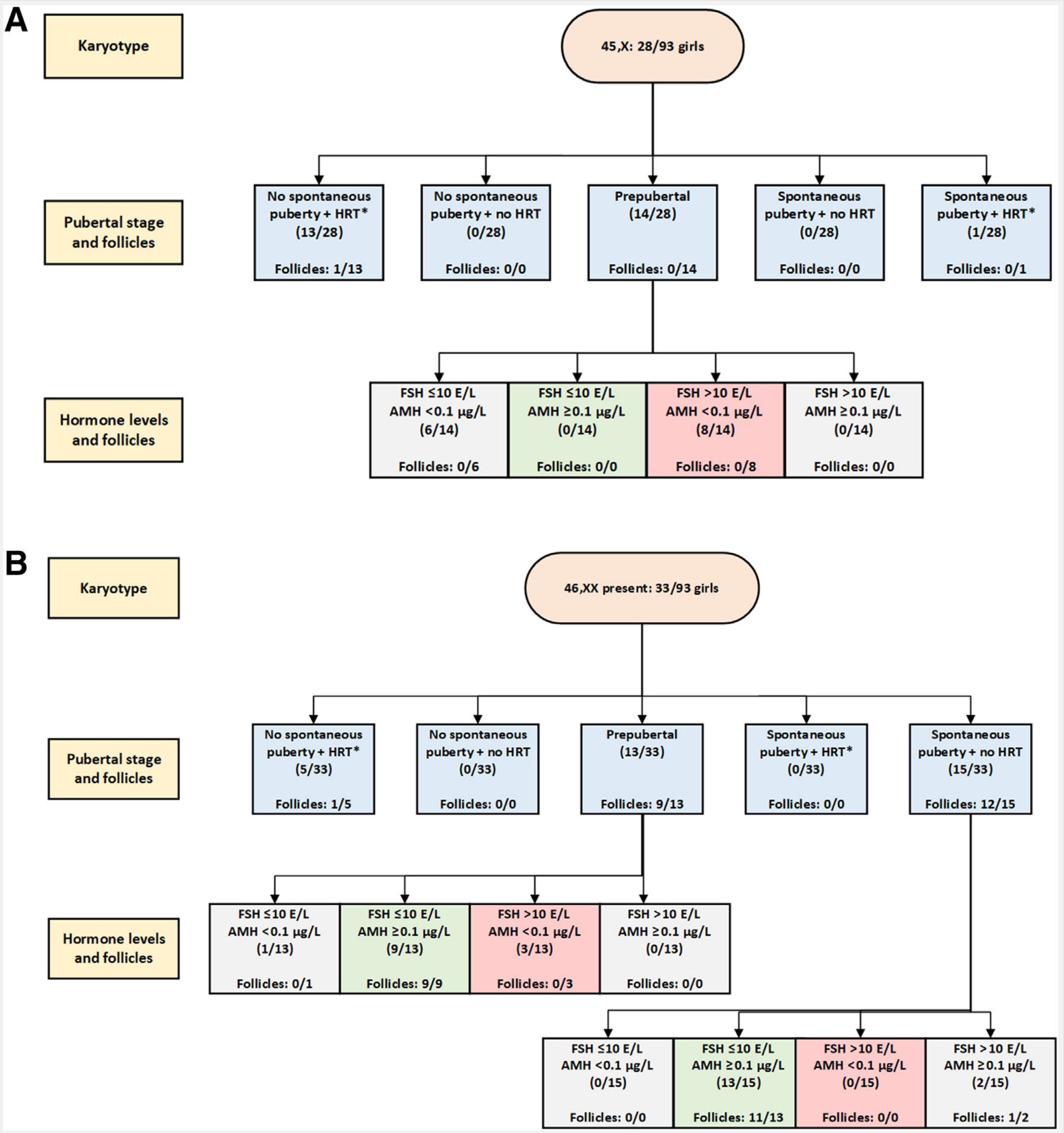
Comparable to previous studies, girls with at least one 46,XX cell line were more likely to have follicles, in their ovarian cortex tissue (17, 30). In our cohort, only one girl in the 45,X group had follicles and she was the only one in that group with AMH levels (≥0.1 μg/L). Additional karyotyping of the urine and ovarian cells of this girl was performed and showed a hidden mosaicism in the ovary, which is most likely the explanation for the presence of follicles in this girl (29).

In line with other studies, spontaneous puberty had a significant positive correlation with the presence of follicles (17, 30, 31). These studies reported that follicles were found in 58%–86% of girls with spontaneous thelarche, 62%–86% with spontaneous menarche, and 10% without spontaneous onset of puberty, which is comparable with our results.

In our cohort, age on its own was not predictive of the presence of follicles. It is known that the ovarian reserve declines as women age, and we hypothesized that the younger the girl, the higher the odds of having follicles (32). The fact that we did not observe this correlation clearly in our study could be explained by our small sample size. Furthermore, our study also showed that the ovarian reserve was already depleted in young girls with TS.

Antimüllerian hormone level had the strongest positive correlation with the presence of follicles in both girls aged <12 years and ≥12 years, and a positive correlation with the number of follicles per ovary. In our study, follicles were found in 89% of the girls with AMH level (≥0.1 μg/L). This is in line with previous studies where a measurable AMH level was predictive in 64%–100% of TS girls with follicles (17, 19, 30). Although these studies have mentioned that

FIGURE 2

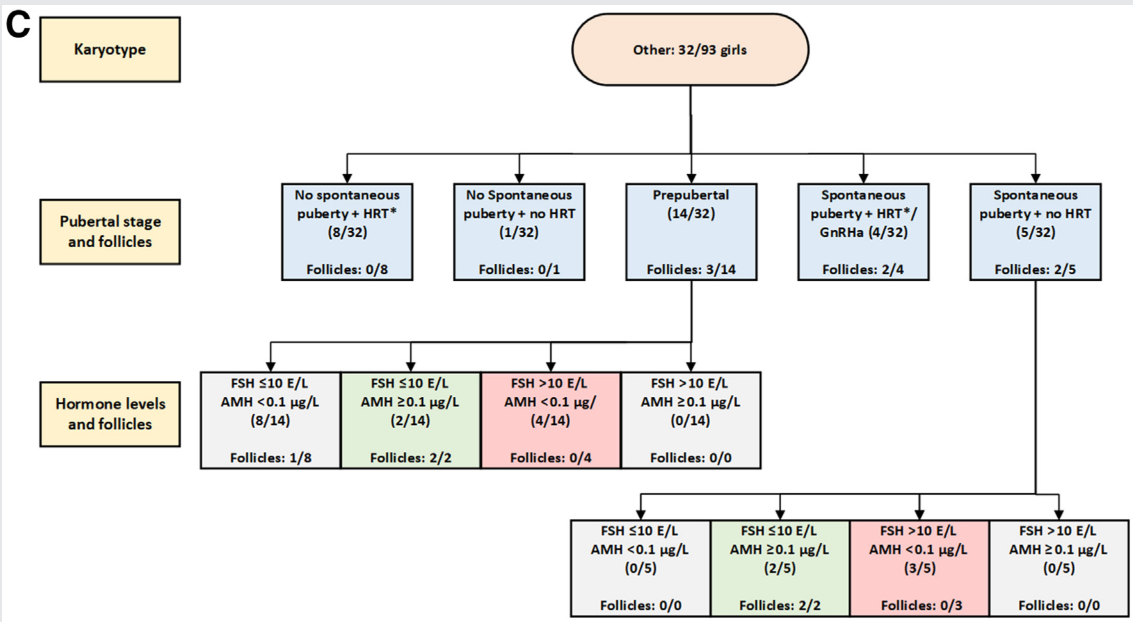


Overview of karyotypes, puberty status, hormone levels, and the presence of follicles in girls with Turner syndrome. Both prepubertal and pubertal girls with a 46,XX cell line present or another karyotype are more likely to have follicles in their ovarian cortex tissue when AMH ($\geq$0.1 μg/L) and FSH ($\leq$10 E/L) levels are present in the blood (green box). No follicles were found in girls with FSH levels (>10 E/L), and AMH levels (<0.1 μg/L) (red box). *, hormone levels of girls receiving HRT or GnRHa were excluded. Other karyotypes with follicles included girls with: 45,X/47,XXX (n = 1), isochromosome (n = 1), X chromosome deletion (n = 1), ring chromosome (n = 2), deletion of one X chromosome and additional part of the other X chromosome (n = 1), and pseudoisodicentric X chromosome (n = 1). Other karyotypes with no follicles included girls with: isochromosome (n = 10), X chromosome deletion (n = 4), ring chromosome (n = 2), pseudoisodicentric X chromosome (n = 1), derivative X chromosome (n = 3), and isodicentric X chromosome (n = 5).

Nadesapillai. OTC in young girls with TS. Fertil Steril 2023.

AMH level could be a promising marker for the presence of follicles in girls with TS, we have seen that follicles were present also in girls with an AMH level (<0.1 μg/L). It is important to notice that in every study, different AMH assays, detection levels, and cutoff values were used. Furthermore, AMH levels may vary in the same patient because of intertest variability, which makes it difficult to compare and interpret AMH levels adequately (33, 34). Therefore, interpretation of

FIGURE 2 Continued



Nadesapillai. OTC in young girls with TS. Fertil Steril 2023.

AMH levels for counseling should be used with caution, preferably after several serial measurements and in combination with other markers.

Besides AMH levels, FSH levels ($\leq$ 10 E/L) also had a positive correlation with the presence of follicles. Follicles were found in 61% of the girls with a FSH level ($\leq$10 E/L) (including prepubertal girls), compared with 50%–100% of girls in other studies. FSH levels ($\leq$ 10 E/L) in prepubertal girls should be interpreted with caution, as this marker is normally low in this group.

Furthermore, we have observed that an inhibin B level ($\geq$ 10 ng/L) had a strong positive correlation with the presence of follicles, comparable to AMH levels. This could be explained by the fact that both AMH and inhibin B are produced by granulosa cells. However, AMH level is considered a more accurate and consistent marker for the ovarian reserve, as inhibin B level is known to fluctuate during the menstrual cycle (35, 36).

The visibility of the ovaries in girls with TS showed a moderate correlation with the presence of follicles. Achieving clear imaging of the ovaries through an abdominal ultrasound is challenging and depends, among other things, on the experience of the sonographer, bladder filling, and ovarian activity. One study investigated the size of the ovaries and antral follicle count in girls with TS aged 11–24 years using ultrasound and magnetic resonance imaging (MRI) (37). In this study, only 37% of girls with TS had one or both ovaries detected using ultrasound, whereas MRI could detect ovaries in 55% of the girls. Distinguishing between a normal small ovary and a streak ovary using MRI was not possible, and there was still a possibility that very small ovaries were not detected using MRI and ultrasound. Overall, there is currently no hard evidence available to differentiate between girls who may or may not benefit from OTC using either ultrasound or MRI.

Previous studies reported follicle density as a representation of the ovarian reserve. We chose to report the ovarian reserve as the calculated number of follicles per ovary, as the size of the ovaries varied widely between girls with TS. However, it should be kept in mind that follicles are heterogeneously distributed in the ovarian cortex, so the follicle density in one tissue fragment may not be representative for all fragments (38).

The calculated number of follicles per ovary in girls with TS varied between 33 and 80.000 follicles. Girls with TS have a significantly lower ovarian reserve compared with girls without TS aged between 2 and 18 years, where the estimated number of follicles per ovary varies between 250.000 and 500.000 (39). It is known that approximately 10% of follicles will perish during the cryopreservation and thawing procedures and that in the first days after autotransplantation, >50% of primordial follicles are lost because of ischemia (40, 41). Given the low number of follicles that were found in girls with TS, the question arises whether enough follicles will remain after autotransplantation of frozen-thawed ovarian tissue to achieve pregnancy.

The quality of the follicles in girls with TS is an important concern as well. In our study, follicles with abnormal morphology were found (e.g., irregular shape of granulosa cells), which is observed also by Mamsen et al. (17). As part of the TurnerFertility trial, the X chromosomal content of ovarian cells was analyzed, as were the possible consequences of aneuploidy in these cells on folliculogenesis (25, 42–44). Follicles of 13 girls with numerical aberrations were karyotyped using FISH, which showed that 92% of the oocytes had a normal X chromosomal content, whereas granulosa cells and stromal cells were mainly aneuploid. The functional consequences of aneuploid granulosa and stromal cells on folliculogenesis were further explored recently using a murine xenograft model (44). It was reassuring to observe that primordial follicles from girls with TS underwent folliculogenesis in this model, but it also showed that the follicle density in pubertal girls was significantly lower than in prepubertal girls after xenografting. Therefore, prepubertal girls with TS could have a more favorable outcome after autotransplantation than pubertal girls.

Strengths and limitations

This study is currently the largest prospective study on OTC in girls with TS worldwide. It was designed primarily because parents and girls with TS were frequently asking about fertility preservation, and evidence on this topic was still lacking. In the current study, a unique and safe setting was created in which almost 100 girls with TS underwent OTC experimentally (45). As pregnancies are described in women with a 45,X karyotype, we decided to include all TS karyotypes in the study to investigate which girls with TS could benefit from this treatment (8, 46). Furthermore, information about the study was provided on multiple occasions and on various media platforms to properly inform families about the study. In contrast to previous studies, a long-term follow-up is integrated with LBR as the primary outcome.

A limitation of this study is that it still involves a relatively small number of participants with a wide variety of karyotypes and phenotypic characteristics because of the limited number of participants allowed by the ethical committee. It was not possible to create a prediction model because only 30 girls had follicles. Furthermore, we still have to wait several years to determine whether OTC results in live-born children in this group. Finally, a volunteer bias may have occurred, as counseling included a prognosis regarding the likelihood of finding follicles in the ovaries, and therefore more girls with favorable parameters may have chosen to participate.

Recommendations for clinical practice

OTC in girls with TS remains an experimental treatment. An international expert panel stated that OTC may be offered to all girls with TS, but only in a research setting with proper counseling about the expectations, risks, and possible benefits of the procedure (45). It is advised to provide fertility preservation counseling in multiple phases (23). Cardiac screening before fertility counseling is strongly recommended to fully inform parents and girls with TS about pregnancy risks. Furthermore, attention must be paid to the mental condition of girls with TS during the whole process. It is recommended to offer psychological support during counseling and further on when needed.

We observed that a 46,XX cell line can be overlooked when only one peripheral cell line is karyotyped. We therefore strongly recommend clinicians to karyotype an additional cell line in girls with a 45,X karyotype in lymphocytes. Furthermore, a combination of karyotype and measurements of several serial AMH and FSH levels could be helpful for clinicians in routine care to provide a prognosis on the ovarian reserve of a girl with TS.

Future perspectives and research

The impact of a unilateral ovariectomy on girls with TS is still unknown. In the follow-up of this trial, girls with TS will be monitored for puberty development and hormone levels after unilateral ovariectomy. Live birth rates of women with TS after transplantation of frozen-thawed ovarian tissue in adulthood will be reported as the second part of the TurnerFertility trial in the future. Furthermore, gene expression profiling of follicles and stromal cells from girls with TS will be performed to gain more insight into the process of premature ovarian failure in girls with TS.

Further research on in vitro maturation is recommended. This technique could be valuable for girls with TS, considering their low follicle density and additional loss of follicles because of the transplantation process. Finally, international collaborations with other research groups within the field of fertility preservation and TS are highly encouraged to further investigate which diagnostic tools can help determine which girls with TS may benefit from fertility preservation.

In conclusion, our study provides insights into which girls with TS are most likely to have follicles in their ovarian cortex tissue. Favorable predictive parameters include either a 46,XX cell line in lymphocytes and buccal cells, spontaneous onset of puberty, or a combination of AMH concentrations ($\geq$0.1 μg/L) and FSH concentrations ($\leq$10 E/L). Karyotyping of two peripheral cell lines is recommended to detect hidden mosaicisms.

It remains uncertain whether girls with TS can become pregnant after autotransplantation of their cryopreserved tissue, considering their low ovarian reserve and significant loss of follicles during the cryopreservation, thawing, and autotransplantation processes. Therefore, OTC should be offered with caution to girls with sufficient ovarian reserve and AMH levels, as well as only in a research setting. During the entire process, attention should be paid to the mental health of girls with TS, and psychological support should be offered when needed.

DECLARATION OF INTERESTS

S.N. has nothing to disclose. J.V.D.V. has nothing to disclose. S.V.D.C. has nothing to disclose. M.S. has nothing to disclose. A.S. has nothing to disclose. M.S. reports Board member special interest group (SIG) Fertility Preservation of Dutch Society Obstetric and Gynaecology (NVOG) and Board member SIG Pediatric and Adolescent Gynaecology (DutchPAG) of

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Ensayo TurnerFertility: preservación de la fertilidad en niñas con Síndrome de Turner por congelación de tejido de corteza ovárica: un estudio prospectivo intervencional.

Objetivo: Evaluar qué niñas con síndrome de Turner (TS) podrían beneficiarse de la preservación de la fertilidad mediante criopreservación de tejido ovárico en base al cariotipo, al estado de la pubertad y a los valores hormonales.

Diseño: Estudio prospectivo intervencional; los participantes fueron incluidos entre 2018 y 2020.

Lugar: Hospital terciario en los países bajos.

Pacientes(s): En total, se incluyeron 106 niñas con TS de edades comprendidas entre 2 y 18 años. Niñas con deleciones menores del cromosoma X, contenido de cromosoma Y, infecciones activas o contraindicaciones para la cirugía fueron excluidas.

Intervención(es): Se realizó una ovariectomía unilateral laparoscópica para obtener tejido cortical ovárico para criopreservación. Un fragmento de tejido por participante se utilizó para determinar el número de folículos por ovario mediante cortes en serie y tinción. Se realizó análisis cromosómico de linfocitos y células bucales. Se tomó una muestra de sangre antes de la ovariectomía para análisis hormonal.

Principal(es) medida(s) de resultado(s): La presencia de folículos en el tejido de la corteza ovárica de niñas con TS en relación con el cariotipo, el estado de la pubertad y los valores hormonales.

Resultado(s): Se realizó una ovariectomía unilateral en 93 niñas con TS. Se produjeron complicaciones después de la cirugía en 5 niñas, incluida luxación o síntomas psicológicos en 2 niñas. En 13 (14%) niñas, se encontró una línea celular 46,XX en las células bucales que estaba ausente en los linfocitos. Se observaron folículos en 30 (32%) de las 93 niñas y se encontraron principalmente en niñas con una línea celular 46,XX en linfocitos o células bucales (Coeficiente Phi = 0.55). El inicio espontáneo de la pubertad (coeficiente Phi = 0.59), la hormona antimülleriana (AMH; correlación biserial puntual [r] = 0.82), los niveles de inhibina B (r = 0.67) y la hormona estimulante del folículo (r = 0.46) también se correlacionaron fuertemente con la presencia de folículos. Además, los niveles de AMH tuvieron una correlación significativa con el número de folículos por ovario (r = 0.66).

Conclusión(es): Los marcadores predictivos favorables para la presencia de folículos incluyeron una línea celular 46,XX, el inicio espontáneo de la pubertad, o una combinación de AMH medible y niveles normales de hormona estimulante del folículo. Se recomienda cariotipar dos líneas celulares periféricas en niñas con TS para revelar mosaicismos ocultos. La criopreservación del tejido ovárico debe ofrecerse con precaución en un contexto de investigación a aquellas con una reserva ovárica suficiente, considerando la pérdida significativa de folículos después de la criopreservación del tejido ovárico y autotrasplante. Los médicos deben prestar atención a la salud mental de las niñas durante todo el proceso.