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ARTICLE

Reference standards for follicular density in ovarian cortex from birth to sexual maturity

BIOGRAPHY

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KEY MESSAGE

Assessing ovarian reserve in patients undergoing ovarian tissue cryopreservation is essential to predict the usability of their tissue in future fertility restoration. Reference standards were developed for cortical follicular density for ages 0–25 years and associated Z-score cut-offs for normal and reduced ovarian reserve. Adoption of Z-scores into fertility preservation programmes is recommended.

ABSTRACT

Research question: Are age-normalized reference values for human ovarian cortical follicular density adequate for tissue quality control in fertility preservation?

Design: Published quantitative data on the number of follicles in samples without known ovarian pathology were converted into cortical densities to create reference values. Next, a sample cohort of 126 girls (age 1–24 years, mean ± SD 11 ± 6) with cancer,

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KEYWORDS

Child
Follicular density
Female fertility preservation
Ovarian tissue cryopreservation
Young adult
Z-score

severe haematological disease or Turner syndrome were used to calculate Z-scores for cortical follicular density based on the reference values.

Results: No difference was observed between Z-scores in samples from untreated patients (0.3 ± 3.5, $n = 30$) and patients treated with (0.5 ± 2.9, $n = 48$) and without (0.1 ± 1.3, $n = 6$) alkylating chemotherapy. Z-scores were not correlated with increasing cumulative exposure to cytostatics. Nevertheless, Z-scores in young treated patients (0–2 years -2.1 ± 3.1, $n = 10$, $P = 0.04$) were significantly lower than Z-scores in older treated patients (11–19 years, 2 ± 1.9, $n = 15$). Samples from patients with Turner syndrome differed significantly from samples from untreated patients (-5.2 ± 5.1, $n = 24$, $P = 0.003$), and a Z-score of -1.7 was identified as a cut-off showing good diagnostic value for identification of patients with Turner syndrome with reduced ovarian reserve. When this cut-off was applied to other patients, analysis showed that those with indications for reduced ovarian reserve ($n = 15$) were significantly younger (5.9 ± 4.2 versus 10.7 ± 5.9 years, $P = 0.004$) and, when untreated, more often had non-malignant haematologic diseases compared with those with normal ovarian reserve ($n = 24$, 100% versus 19%, $P = 0.009$).

Conclusion: Z-scores allow the estimation of genetic- and treatment-related effects on follicular density in cortical tissue from young patients stored for fertility preservation. Understanding the quality of cryopreserved tissue facilitates its use during patient counselling. More research is needed regarding the cytostatic effects found in this study.

INTRODUCTION

Female fertility is dependent on the number of immature primordial follicles in the ovary, also referred to as the ovarian reserve. The number of primordial follicles peaks mid-gestation when the non-renewable ovarian reserve is established, and then continues to decrease until menopause at around 50 years of age (*Faddy and Gosden, 1996*). The controlled recruitment and survival of primordial follicles can be disturbed by genetic or endocrine disorders, such as Turner syndrome, as well as medical interventions and toxic exposures, such as chemotherapy, irradiation or environmental pollution, leading to partial or complete depletion of the ovarian reserve prematurely (*Björvang et al., 2021*; *Borgström et al., 2009*; *Hanson et al., 2017*; *Pampanini et al., 2019*; *Weiss and Clapauch, 2014*).

Ovarian tissue cryopreservation (OTC) is a fertility-saving option for patients subjected to gonadotoxic treatments (*Burns et al., 2018*). The cryopreserved ovarian cortical tissue retains its endocrine functionality, and, when thawed and auto-transplanted, it is able to restore ovarian function in otherwise menopausal and infertile patients (*Donnez and Dolmans, 2017*). Knowledge regarding follicular density in cryopreserved cortical samples is of great value when estimating the quality and future fertility potential of the tissue. However, the impact of the age-dependent decrease in ovarian reserve needs to be controlled in order to identify the possibly deviating follicular count correctly due to adverse effects of severe disease and gonadotoxic exposures.

This study aimed to develop a simple, cohort-independent method for normalizing age-dependent differences in the number of follicles in ovarian cortical samples collected for fertility preservation. Published quantitative histological studies on human ovarian reserve were reviewed, numbers of follicles were converted into cortical follicular densities, and age-normalized reference mean values from birth to puberty were calculated. The performance of the model was evaluated using Z-scores based on follicular densities in cryopreserved fertility preservation samples from the Sveafertil study and Helsinki Children's Hospital (HCH) cohort, as well as previously published quantitative histological data from patients with Turner syndrome and haemoglobinopathies (*Hreinsson et al., 2002*; *Mamsen et al., 2021*, *2019*).

MATERIALS AND METHODS

Ethical approval and informed consent

Patients were offered OTC if they were at high or very high risk of iatrogenic premature ovarian insufficiency due to planned treatments (allogenic/autologous haematopoetic stem cell transplantation or radiotherapy with ovary in the field), in accordance with the guidelines on fertility preservation of the Nordic Society of Paediatric Haematology and Oncology (http://www.nopho.org) and Swedish national recommendations for infertility risk assessment (https://vavnad.se/wp-content/uploads/2016/11/framjande-av-reproduktionsformaga-unga-v1-00.pdf). OTC was offered as part of a research protocol in children approved by the Swedish Ethical Review Authority (Sveafertil patients, Dnr:2019-03802, date of approval 23 September 2019) and by the Ethics Committee of Helsinki University Hospital (HCH patients, Dnr 340/13/03/03/2015, date of approval 22 October 2015). All samples were pseudonymized with random codes before access and start of analysis. All age-appropriate patients or guardians provided their written informed consent for participation in the study. Parental written consent was obtained from all patients aged <18 years. The work was carried out in accordance with the Declaration of Helsinki.

Patient cohorts

Analysed data were derived from ovarian cortical samples from 13 patients (age 2–16 years) from the Sveafertil study in Sweden (sveafertil.ki.se) and 38 patients (age 2–20 years) from the HCH cohort in Finland. All participating patients were diagnosed with cancer or severe haematological disease. Basic characteristics of the patients, including age, diagnosis and cancer therapy prior to OTC, were reported by the treating physicians and are summarized in Supplementary Table 1A. Exposure to alkylating agents was calculated as cyclophosphamide equivalent dose (CED) (*Green et al., 2014*), and exposure to anthracyclines was calculated as the cumulative doxorubicin isotoxic equivalent (DIE) dose (*Shankar et al., 2008*) using the following conversion factors: 1 for doxorubicin, 0.833 for daunorubicin, 5.0 for idarubicin and 4.0 for mitoxantrone (*Funke et al., 2021*).

To test the model in non-cancer patient groups, previously published data on follicular densities from freshly fixed ovarian cortical tissue in 18 patients with haemoglobinopathies (age 3–17 years) (*Mamsen et al., 2021*) and 24 patients with Turner syndrome (age 5–22 years) were included (*Hreinsson et al., 2002*; *Mamsen*

et al., 2019). Follicular counts of all patients with haemoglobinopathies and 15 patients with Turner syndrome (Mamsen et al., 2019) were retrieved from either 5- or 30-μm sections of ovarian cortical tissue (Mamsen et al., 2021, 2019). Follicular counts in the remaining 10 patients with Turner syndrome were retrieved from 4-μm sections (Hreinsson et al., 2002). The demographics of these patients have been published previously and are summarized in Supplementary Table 1C. Finally, the authors' previously published data on follicular densities from frozen-thawed ovarian tissue retrieved from 4-μm sections in 33 patients (age 1–24 years) with cancer or haemoglobinopathies who were not included in the presently analysed material, were included (Azarbaijani et al., 2015). The demographics of these patients are summarized in Supplementary Table 1B.

Tissue processing and histological analysis

A small piece of every ovarian sample from the Sveafertil study and HCH fertility preservation programme was separated from the tissue that was cryopreserved, and freshly fixed in either formalin or Bouin's solution for quality control prior to cryopreservation. The fixed tissue was embedded in paraffin, serially sectioned (4 μm), stained with haematoxylin and eosin, and digitalized using slide scanner (Pannoramic Scanner, 3DHistech, Budapest, Hungary). Every 10th section was evaluated using Pannoramic viewer virtual microscope software (Version 1.15.4, 3DHistech). Morphological assessment was conducted on a mean area of 3.6 mm^2 (range 0.4–33.2 mm^2) of the tissue with the following criteria: (i) follicular count and cortical surface area were assessed within 1 mm of the surface epithelium; (ii) follicles were counted from primordial to primary maturation stage, seen morphologically as an oocyte with a maximum of one cuboidal granulosa layer (i.e. unilaminar follicles); (iii) follicles with or without a nucleus were counted; (iv) mean oocyte diameter of the largest 10% of follicles was recorded by taking two perpendicular measurements, using the oocyte membrane as a reference; and (v) all follicles were counted regardless of their morphological quality. Two blinded observers evaluated a subset of six sections independently. These samples were comprised of three to seven serial sections with eight to 150 follicles per section. The results showed interobserver concordance >97%. Next, both observers counted a random selection of the samples. Representative images of ovarian tissue included in this study are shown in Supplementary Figure 1.

As the diameter of follicles (>35 μm) was larger than the thickness of the sections (4 μm), each follicle appears in several sections. To avoid multiple counting of the same follicle, a modified version of the formula presented previously by Schmidt et al. was used, in addition to assessing every 10th section (Schmidt et al., 2003). In the modified formula, the mean oocyte diameter of the largest 10% of follicles was considered, to correct for the probability of counting the same follicle in multiple sections. For each sample, the total number of follicles and the total volume of the cortex were extrapolated from the selected sections, and follicular density was calculated based on these values, as explained in detail in the Supplementary Materials and Methods.

Data extraction for reference standards

Reference values for the Z-score calculation were based on previously published quantitative histological studies used by Wallace and Kelsey (2010) in their model of human ovarian reserve from conception to menopause. Follicular counts in all reports used a similar definition of non-growing follicles as proposed by Block (1952) and Gougeon and Chainy (1987). All follicles, regardless of their morphological quality, were noted. Data were gathered from reports that had comparable numbers of follicles (either follicular count or follicular density) and age presented unambiguously. Only data from individuals aged 0–25 years (n = 60) from four previous reports were used (Baker, 1963; Block, 1953, 1952; Hansen et al., 2008). Numbers of follicles were collected and a mean value for one ovary was calculated for each individual, as displayed in Supplementary Table 2. Data were pooled into a single dataset and divided into age groups to establish the reference values.

To determine the mean ovarian volume for each age group, a previously published validated model of ovarian volume throughout life was used (Kelsey et al., 2013). The ellipsoid volume formula (1/6 * π * A * B * C) was used where A, B and C are the length, height and depth of an ovary, with an estimation that B = C = 0.6 × A which approximates the values for the human ovary (Mclaughlin et al., 2015). The ovarian cortical volume was estimated as 1 mm from the ovarian surface, as reported previously (De Roo et al., 2019). Estimated mean cortical volumes and proportion of cortex of ovarian volume are shown in Supplementary Table 3.

Reference follicular density values (follicles/cm^3 of cortex) based on the number of follicles and estimated cortical volume (including mean and SD values) for age groups 0–25 years are presented in Supplementary Table 4. The reference data were divided into six age groups (0–2, 3–5, 6–10, 11–14, 15–19 and 20–25 years) based on the availability of reference samples and biological similarities, such as expected pubertal status. To calculate the reference values for the age group 3–5 years, the average values of previous and subsequent age groups were used as there were no reference values available. The follicular density values were log-transformed using $\log_e$ transformation described by Higgins et al. (2008) to obtain normally distributed data, and the reference mean and SD were calculated for each age group (Supplementary Table 4). The reference model, shown as non-transformed cortical follicular density by age, is displayed in Supplementary Figure 2.

Z-score calculation for the study cohort samples

Z-scores for each patient in the study cohort were calculated using logarithmic values of the patient's follicular density (Supplementary Table 1), and the mean and SD of the respective age groups in the reference model (Supplementary Table 4) according to the following formula:

$$Z = \frac{x - M}{SD}$$

where Z = Z-score, x = natural logarithm of follicular density of the study sample, $\log_e$ (follicular density), M = mean follicular density for the respective age group in the reference data, and SD = reference SD for the respective age group. The study cohort consisted of newly collected samples (Sveafertil and HCH) as well as previously published samples [girls with haemoglobinopathies (Mamsen et al., 2021), girls with Turner syndrome (Hreinsson et al., 2002; Mamsen et al., 2019) and the present authors' previous follicular density data of patients with cancer or haematologic disease (Azarbaijani et al., 2015)].

Statistical analysis

Statistical analysis was conducted in R (R Core Team, 2014, Version 4.1.1), and

figures were produced using ggplot2 (*Wickham, 2016*, Version 3.3.6). The comparisons of patient background variables, follicular density and Z-scores between groups were undertaken using Kruskal–Wallis test, Wilcoxon test and Fisher's exact chi-squared test. As no difference was found in Z-scores between the authors' previously published samples (Asadi *Azarbaijani et al., 2015*) and the newly analysed samples, the materials were combined ($n = 84$). The dataset was then divided into: (i) untreated patients with severe haematologic disease and cancer; (ii) treated patients with cancer exposed to non-alkylating chemotherapy; and (iii) treated patients with cancer exposed to alkylating chemotherapy (Supplementary Table 1). Spearman correlation coefficient (r_s) was used to determine the relationship between CED, DIE dose, age at procedure and Z-score. Z-scores were further entered as dependent variables and DIE dose, CED and age as independent variables in multiple linear regression analysis. Receiver operating characteristic (ROC) curves and area under the curve (AUC) were studied in the different patient groups to estimate the diagnostic value of Z-scores to predict patients with reduced ovarian reserve. All tests of significance were two-tailed, and $P < 0.05$ was considered to indicate significance.

RESULTS

Reference standard values for ovarian cortical follicular density were derived from girls and women up to 25 years of age using published data on follicular counts in human ovaries, and the model was then applied to calculate Z-scores for a patient cohort consisting of patients with cancer, haemoglobinopathies or Turner syndrome. Due to the rarity of material, the cohort was pooled from various sources, as outlined in Supplementary Table 1. Data from the authors' patient cohorts from newly and previously analysed samples were pooled. In these data, the distribution of diagnoses and exposure to CED and DIE dose did not differ between the previously reported and newly analysed cohorts. However, the previously reported patients were older (12.8 ± 6.9 versus 7.9 ± 5.2 years, $P = 0.004$). Ages of patients with haemoglobinopathies and Turner syndrome did not differ significantly from the patient cohort. Cohort demographics and characteristics are summarized in TABLE 1.

Follicular densities in the individual patient samples are presented in Supplementary Table 5 and in FIGURE 1 overlaid with the reference model. The calculated Z-scores by patient group are displayed in FIGURE 2 and TABLE 1. All values for individual patients can be found in Supplementary Table 5. The majority of the Z-scores in the patient cohort were within ± 3 SD of the reference mean value (FIGURE 2). There were no significant differences in Z-scores between the untreated samples (0.33 ± 35, $n = 30$) and samples exposed to alkylating or non-alkylating agents (0.48 ±

TABLE 1 CLINICAL CHARACTERISTICS, FOLLICULAR DENSITY AND Z-SCORES IN THE DIFFERENT PATIENT GROUPS OF THE SAMPLE COHORT

Characteristic		Data from authors[a]			Data published by others	
		Untreated	Non-alkylating chemotherapy	Alkylating chemotherapy	Haemoglobinopathies[b]	Turner syndrome[c]
n		30	6	48	18	24
Age (years)	Mean ± SD	13.5 ± 5.8	4.1 ± 1.8[e]	8.3 ± 5.7[e]	10.4 ± 4.2	15.2 ± 3.8
	Range	3.7–24	1.7–6	1–20	2.8–17.4	5–22.4
	P-value	*<0.001*	0.003	0.001	0.09	0.4
Follicular density (n/mm^3)	Mean ± SD	480 ± 576	1193 ± 1011[d]	970 ± 937[d]	465 ± 542	78 ± 146[f]
	Range	1–2108	289–2970	11–3449	22–1231	1–519
	P-value	*<0.001*	0.04	0.02	0.7	<0.001
CED (mg/m^2)	Mean ± SD	n/a	n/a	7602.4 ± 7484.1	n/a	n/a
	Range			0–39,593		
DIE dose (mg/m^2)	Mean ± SD	n/a	88.3 ± 138.6	158.8 ± 107.5	n/a	n/a
	Range		0–300	0–450		
	P-value			0.18		
Z-score	Mean ± SD	0.3 ± 3.5	0.1 ± 1.3	0.5 ± 2.9	0.2 ± 1.8	-5.2 ± 5.1[f]
	Range	−12.3 to 5.3	−1.9 to 1.5	−9.7 to 5.4	−2.3 to 3.5	−13.6 to 2.7
	P-value	*<0.001*	0.47	0.87	0.46	<0.001

P-values depicted in italic in the untreated column were calculated by Kruskal–Wallis post-hoc analysis; all other *P*-values are exact values as compared with untreated patients, calculated by pairwise Wilcoxan test.

[a] Combined from 51 newly analysed samples and 33 samples reported previously by *Azarbaijani et al. (2015)*.

[b] Reported by *Mamsen et al. (2021)*.

[c] Reported by *Mamsen et al. (2019)* and *Hreinsson et al. (2001)*.

[d] $P<0.05$.

[e] $P<0.01$.

[f] $P<0.001$ compared with untreated patients.

CED, cyclophosphamide equivalent dose; DIE, doxorubicin isotoxic equivalent dose.

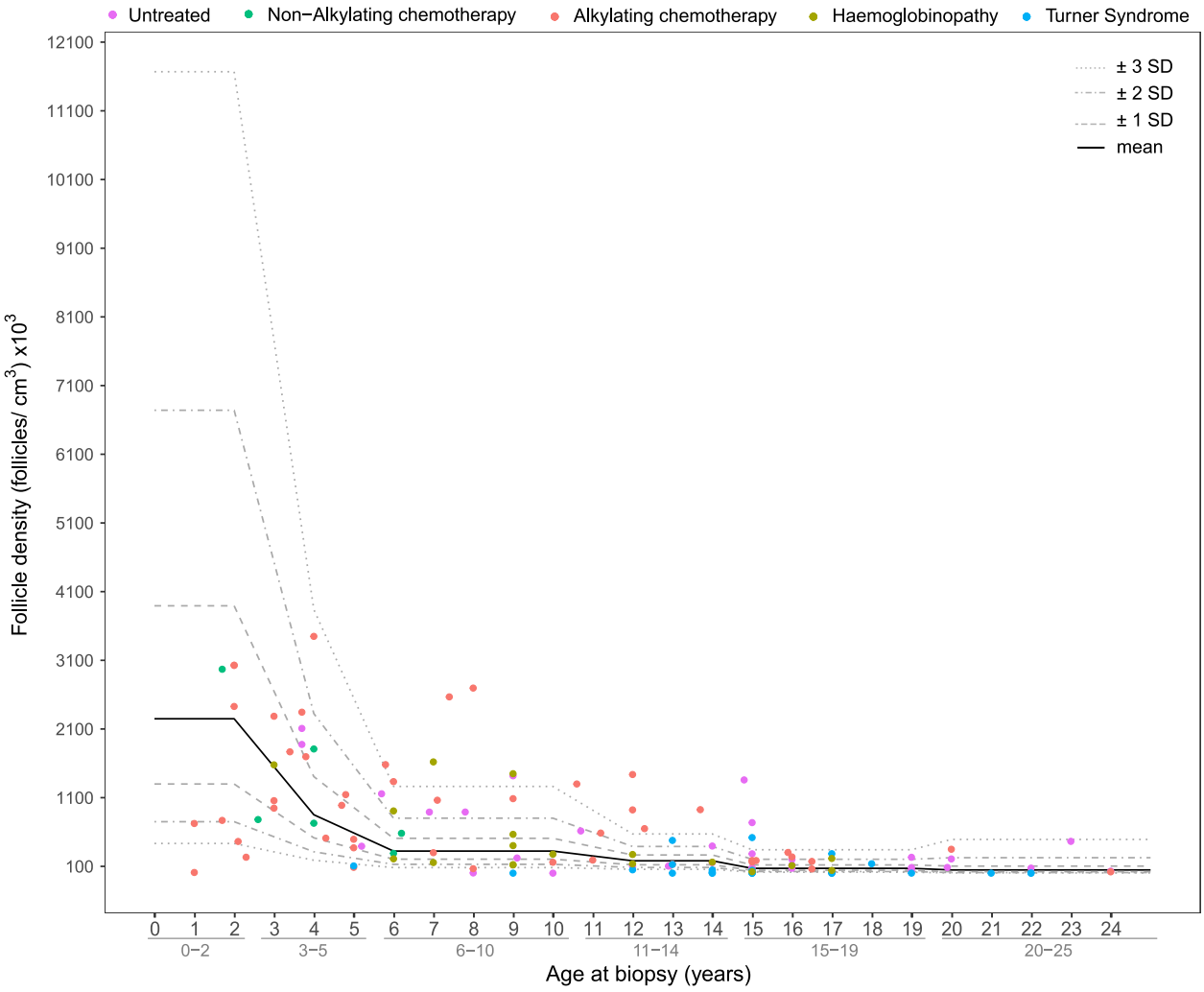

FIGURE 1 Follicular density in fertility preservation material. Follicular density is presented by age in untreated samples (n = 30), samples with non-alkylating (n = 6) and alkylating (n = 48) chemotherapy from cancer patients, and samples from patients with haemoglobinopathies (n = 18) and Turner syndrome (n = 24). Data points represent individual patients. Mean and SD shown for reference follicular density for human ovarian reserve.

2.9, n = 48, P = 0.87; 0.14 ± 1.3, n = 6, P = 0.47, respectively). The mean Z-score in samples from patients with Turner syndrome (-5.21 ± 5.1, n = 24) were significantly lower compared with samples from untreated patients (P = 0.0003) (FIGURE 2, TABLE 1). The possible effect of mosaicism on the Z-scores was further studied. Samples from patients with mosaic (n = 11) and monosomic (n = 13) Turner syndrome showed equally low Z-scores (-5.28 ± 6.2 versus -5.16 ± 4.3, P = 0.96). Also, the Z-scores in patients with haemoglobinopathies (-0.15 ± 1.8, n = 18) were comparable with those of untreated patients and patients exposed to chemotherapy (FIGURE 2, TABLE 1).

Next, the relationships between age, treatment and Z-scores were studied using Spearman correlation. Younger age at OTC (r_s = 0.430, df = 52, P = 0.001) correlated significantly with decreasing Z-score values among patients who were exposed to chemotherapy, whereas higher exposure to CED (r_s = 0.042, df = 52, P = 0.760) and DIE dose (r_s = 0.043, df = 52, P = 0.758) did not (n = 54). In support of this, patients in the youngest age group (0−2 years) who were exposed to chemotherapy had a lower mean Z-score (-2.09 ± 3.1, n = 10, P = 0.04) compared with other exposed age groups (11−14 years, 2.81 ± 2.3, n = 7; 15−19 years, 1.35 ± 1.3, n = 8; both P = 0.04) (FIGURE 3). When untreated patients (n = 30) were studied, no correlation was found between Z-score and age (r_s = -0.192, df = 28, P = 0.270) and no differences between age groups were found (FIGURE 3). Multivariate linear regressions to adjust for differences in age at OTC and cumulative exposure to DIE dose and CED were carried out. Age remained the only independent predictor of a lower Z-score (R = 0.473, B = 0.254, P = 0.001).

One of the aims of this study was to determine a cut-off Z-score that would help to identify patients with reduced ovarian reserve. In ROC analyses of the different patient groups within the full cohort (n = 126), a Z-score <-1.7 displayed good diagnostic value for identifying Turner syndrome based on follicular density (AUC 0.82, 95% CI 0.70−0.94, n = 24) with sensitivity of 75% and specificity of 83% (Supplementary Figure 3). In contrast, ROC analyses showed no diagnostic value for identifying patients exposed to alkylating agents based on Z-score among all patients with cancer and severe haematological disease (AUC

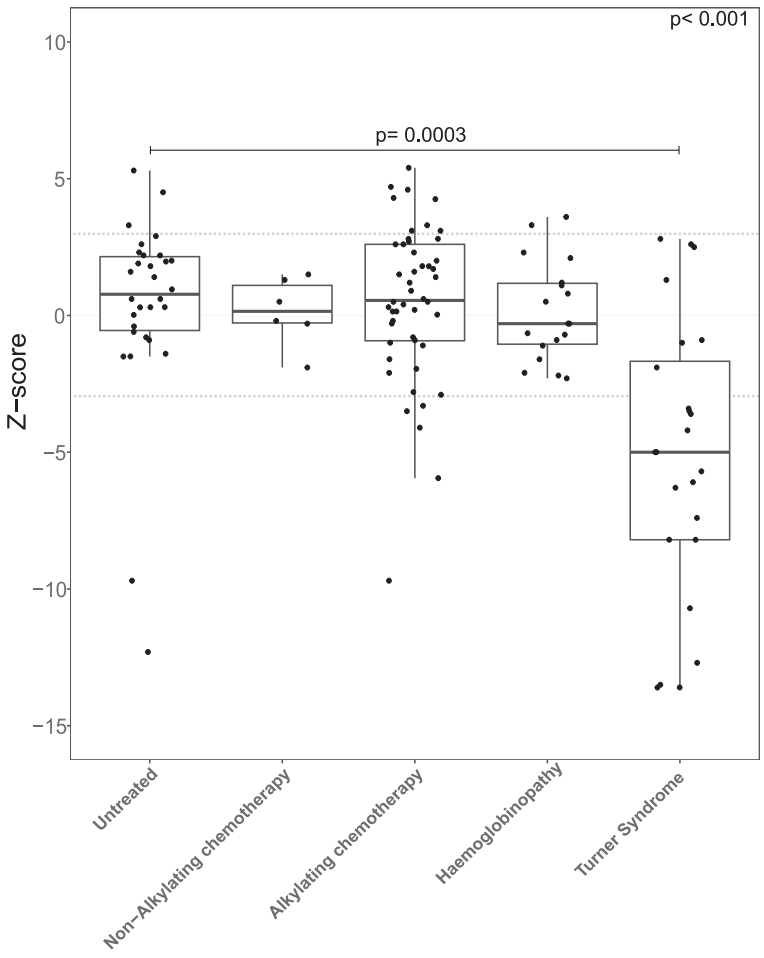

FIGURE 2 Z-scores for follicular density in different patient groups. Boxplots displaying the median, interquartile range and non-outlier range (whiskers) of Z-scores derived from untreated patients (n = 30), patients exposed to non-alkylating (n = 6) or alkylating (n = 48) chemotherapy, and patients with haemoglobinopathies (n = 18) or Turner syndrome (n = 24). Dots represent individual samples. Dotted lines correspond to the Z-score ± 3 SD for reference. Samples of patients with Turner syndrome and untreated patients were compared using Kruskal–Wallis test (top right) with post-hoc pairwise Wilcoxon test.

0.45, n = 102), or among patients exposed to chemotherapy (AUC 0.42, n = 54).

The Z-score cut-off value of -1.7 was used to classify all patients (except those with Turner syndrome) in the cohort as having normal (Z-score > -1.7) or reduced (Z-score ≤ -1.7) ovarian reserve. In total, 15 of the 102 patients were identified to have potential reduced ovarian reserve. The patients with reduced ovarian reserve were significantly younger (5.7 ± 4.2 versus 10.7 ± 5.9 years, P = 0.002) than those with normal ovarian reserve (**TABLE 2**). CED and DIE dose exposure did not differ between the groups. When untreated patients (n = 48) were analysed separately, a higher prevelance of reduced ovarian reserve was found in patients with haematologic diseases compared with patients with cancer (100% versus 44%, P = 0.009) (**TABLE 2**).

DISCUSSION

For prepubutal girls, OTC is the only possible option for fertility preservation prior to high-risk gonadotoxic treatments (*Dolmans et al., 2021*; *Jadoul et al., 2010*; *Muñoz et al., 2016*). This is also the case for older girls and young women if the treatment cannot be postponed to allow time for the collection of mature oocytes after hyperstimulation. Patients in need of fertility preservation are a heterogenous group in terms of age, diagnosis and previous treatment exposures (*Jensen et al., 2011*). These factors can impact the ovarian reserve, and consequently influence the potential outcome of fertility preservation. To date, no models exist to evaluate the normality of follicular density in a piece of ovarian cortical tissue. Therefore, reference standards for follicular density in ovarian cortex were developed that can be used to calculate Z-scores to estimate the normality of the ovarian reserve in patients. This model takes into account the age-dependent decline in the follicular reserve, which is a clear benefit.

Using the model, analysis demonstrated that Z-scores for follicular density in cortical samples derived from untreated OTC patients were within the normal range (± 3 SD, including 99.7% of the reference values) for the respective age groups, confirming the adequate performance of the model. In addition, an independent cohort from another hospital consisting of patients with haemoglobinopathies was used (*Mamsen et al., 2021*), and showed that the Z-scores performed similarly. A primary advantage of using Z-scores is that the analysis of patient samples is independent of age distribution in the study population; every patient can be assigned a Z-score based on the reference standards. Quantification of follicular density in the ovarian cortex standardized by age will allow data from patients with similar genetic diseases or therapy exposures to be combined with larger study populations for meta-analyses. Considering the rarity of childhood cancers and severe haematological diseases, it is a significant benefit to the research community to be able to merge data for larger analyses.

Cumulative alkylating agent exposure has previously been associated with reduced fertility (*Meirow, 2000*; *Sklar et al., 2006*). However, in the present study, no correlation was observed between Z-score and CED exposure dose; patients with cancer exposed to alkylating agents scored similarly to untreated patients and patients exposed to non-alkylating chemotherapy. One explanation could be that CED exposure may not affect the number of follicles directly, but rather the quality of the available follicles, thus leading to reduced fertility. In this study, the quality of follicles in the patient samples was not considered, and all visible follicles were counted, regardless of their morphology. As archived patient samples from different biobanks were utilized, tissue fixation protocols could not be altered. Bouin's solution is widely recognized as the preferred fixation method for morphological evaluation of follicles, as formalin is known to introduce artefacts that can be interpreted as damage in the tissue (*Adeniran et al., 2021*). Old archived

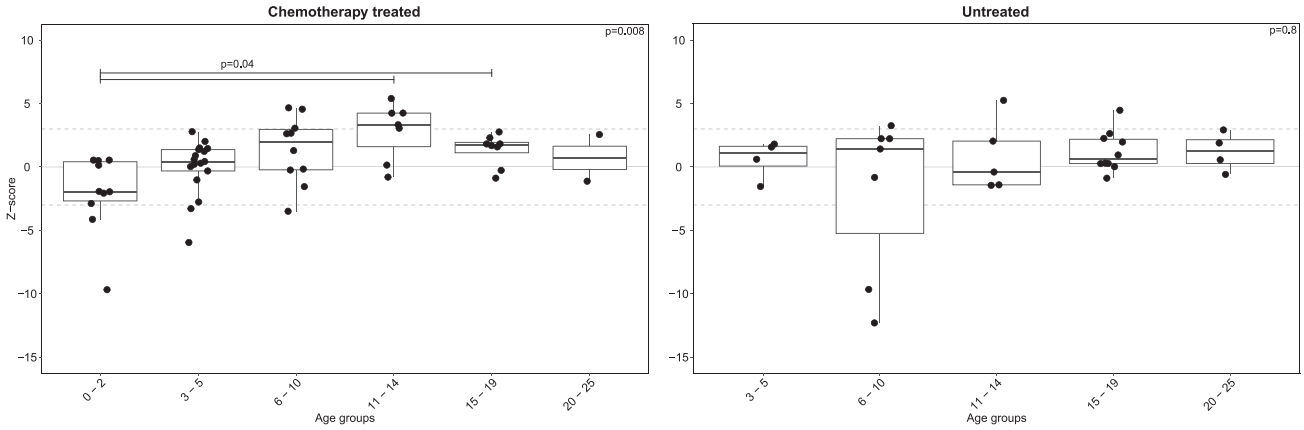

FIGURE 3 Z-score for follicular density in different age groups. Boxplots displaying the median, interquartile range and non-outlier range (whiskers) of Z-scores in patients treated with chemotherapy (n = 54) and untreated patients (n = 30) by age group. Dots represent individual samples. Dotted lines correspond to the Z-score ± 3 SD for reference. Age groups within the treatment type were compared using Kruskal–Wallis test (top right) with post-hoc pairwise Wilcoxon test.

tissue samples are typically formalin-fixed, which prevented detailed analysis of healthy and degenerated follicles in this study. Therefore, the quality of the follicles in the patient samples could not be analysed, and instead focused on all visible follicles regardless of their morphology.

Another explanation could be the age of the patients. The average Z-scores were significantly lower in the youngest girls (0–2 years) exposed to chemotherapy compared with adolescent patients (11–19 years) exposed to chemotherapy, regardless of exposure levels. This is in contrast to earlier studies which suggested less insult to the ovarian reserve in younger patients receiving gonadotoxic treatment compared with adolescent patients (*El Issaoui et al., 2016*; *Schmidt et al., 2010*). This is believed to be due, in part, to the higher number of follicles in young girls. However, this hypothesis has been questioned by previous in-vitro studies suggesting that there may be key differences in follicular recruitment and development between the ovaries of young girls and adults (*Anderson et al., 2014*; *Azarbaijani et al., 2015*). The capacity of primordial follicles to be activated and reach the secondary stage of development may be age-dependent, suggesting decreased in-vitro viability and development of ovarian follicles from very young girls (*Anderson et al., 2014*). In the authors' previous study, older age correlated with fewer follicles, more developing follicles and fewer atretic follicles after culture (*Azarbaijani et al., 2015*). The present results are in line with these observations, as the Z-scores in the youngest exposed prepubertal patients were significantly lower compared with those of exposed adolescent patients. This indicates that follicles in younger patients may be more sensitive to chemotherapy compared with follicles in older patients. Although prepubertal girls may be less prone to premature ovarian insufficiency due to a higher total number of follicles, existing follicles can be more susceptible to atresia after exposure to chemotherapy.

TABLE 2 COMPARISON OF PATIENTS WITH REDUCED AND NORMAL OVARIAN RESERVE

Characteristic	Z-score		*P*-value
	≤-1.7	>-1.7	
n	15	87	
Age (years)	5.7 ± 4.2	10.9 ± 5.9	**0.002**[a]
Malignant diagnosis[b]	10 (67%)	68 (78%)	0.17
Exposed to alkylating chemotherapy	10 (60%)	39 (44%)	0.13
CED (mg/m^2)	8532 ± 7524	7388 ± 7557	0.88[a]
DIE (mg/m^2)	176 ± 95	158 ± 111	0.84[a]
Untreated patients	5 (33%)	43 (49%)	0.12
Non-malignant haematologic disease	5 (100%)	19 (44%)	**0.009**

Patients without Turner syndrome were grouped by Z-score indicating low (≤-1.7) or normal (>-1.7) ovarian reserve, and compared in terms of age, treatment and diagnosis.

Values are depicted as mean ± SD or number and percentage of group total. Significant *P*-values are depicted in bold.

[a] Compared using Wilcoxon test. All other comparisons made using Fisher's exact chi-squared test.

[b] Includes leukaemias and non-benign tumours.

CED, cyclophosphamide equivalent dose; DIE, doxorubicin isotoxic equivalent dose.

Currently, ovarian tissue is collected for fertility preservation of patients of all ages, including 0–2-year-old girls exposed to chemotherapy. To date, no studies have indicated that this tissue results in restoration of endocrine function, and no live births have been reported after auto-transplantation. The youngest patients that have successfully used stored tissue to achieve pregnancy and live birth at a later date were aged 9–14 years at the time of OTC (*Demeestere et al., 2015*; *Matthews et al., 2018*; *Rodriguez-Wallberg et al., 2021*). Further investigations on quality and use of ovarian tissue from very young patients for OTC are required to improve and refine fertility preservation for these patients.

Furthermore, a cut-off mean follicular density Z-score of -1.7 was found to have good diagnostic value for identification of

patients with Turner syndrome. Females with Turner syndrome lack one X chromosome in all (monosomic Turner) or some (mosaic Turner) of their cells, leading to a wide range of adverse health consequences. The main reproductive effect is reduced ovarian reserve and premature ovarian insufficiency (*Viuff and Gravholt, 2022*). This was confirmed by the present study as the mean Z-score in the patients with Turner syndrome was -5.2, reflecting the poor reproductive quality of the tissue collected for fertility preservation. The clinical conclusion of these findings is that a Z-score <-1.7 reflects a significant depletion of the ovarian reserve. Interestingly, when the Z-score cut-off of <-1.7 was applied to other patient groups in this study, 15 patients with potentially reduced ovarian reserve were identified. These 15 patients were significantly younger compared with patients with a Z-score >-1.7, and, if untreated, more often had a non-malignant haematologic diagnosis, often with a genetic background, such as bone marrow failure, red blood cell disorder and immunodeficiency. This result suggests that non-malignant diseases or associated chronic anaemia, iron overload and inflammation *per se* may have an impact on the size of the ovarian reserve. Interestingly, the two extreme outliers with Z-scores of -12.3 and -9.7 were patients diagnosed with β-thalassaemia and DOCK8-related immunodeficiency. The present data strongly encourage further study on the effect of haematologic/immunologic diseases on ovarian reserve, as this could help to identify limitations of OTC in patients with non-malignant conditions.

A limitation of this study is the reliability of reference material obtained from a small number of old quantitative histological studies of whole ovarian tissue from autopsies, contrary to ovarian cortical samples that were used to test the model. The reliability of the reference material is also related to the accuracy of the mathematical conversion of cortical densities. In order to estimate cortical densities, an age-related normative model for the volume of the human ovarian cortex was used, and non-growing follicles in whole ovaries were estimated to be located within 1 mm of the ovarian surface epithelium. It is possible that this difference with regard to the source of the ovarian samples and mathematical conversion influences the findings. The present observation that the calculated volume of the cortex as a proportion of the entire ovary decreased steadily from 63% in the youngest age group (<2 years) to 23–24% among the girls in their mid teens to early 20s is well in accordance with previous histological reports. These reports describe that the cortex comprises 76–93% of newborn and 24% of adult ovarian volume (*Forabosco and Sforza, 2007*; *Mclaughlin et al., 2015*). Patient material was also limited due to accessibility of samples, and six ovarian cortical samples had to be excluded from the analysis due to a lack of cortical tissue in the histological sections. Furthermore, no conclusions could be made about the non-alkylating group, as this group only contained six samples. Finally, in the present study, the quality of follicles could not be analysed, which probably limited correlation between cumulative alkylating agent exposures and Z-score values. In a potentially more advanced future model, folliclular quality needs to be taken into consideration. Strengths of this study include the number of samples of patient material used, and the consecutive nature of the study samples, which provide a good representation of the young patients participating in the fertility preservation programmes (*Jensen et al., 2011*). An additional strength is the inclusion of both newly quantified samples and published datasets from the literature, showing that the model is applicable to datasets collected in different hospitals at different times. This suggests that this tool will enable large meta-analyses.

This study included data on follicular count from girls and women aged up to 24 years to establish the reference values. Of the 126 patients included, 15 were aged 18–24 years. The reference standard values for ovarian cortical follicular density covered ages up to 25 years . At present, OTC is recommended for women with a maximum age of 35–37 years who have an age-appropriate ovarian reserve. Therefore, developing the reference standards further to include these ages is the logical next step. The present study focused on ovarian tissue from young patients because the risk–benefit considerations are more complex in children, as OTC is not a medically established routine for infants. In addition, the ovarian cortex in children contains more follicles, which therefore have a more homogeneous distribution. This facilitates the quantification of follicles from limited tissue biopsies and, accordingly, produces a more robust model. Adult ovaries are known to have a heterogeneous distribution of follicles, and the ovarian reserve is therefore more difficult to quantify. Furthermore, methods for histological quantification of ovarian reserve are not standardized. Earlier studies reported more representative results when multiple biopsies across the ovaries, or even entire ovaries, were quantified morphologically (*Lass, 2001*). Further development of the Z-score approach could include standardization of the histological quantification approach in terms of biopsy number, section number and section spacing, for example. Additionally, including follicular quality in the reference standard model will increase its functionality, and enable researchers and physicians to draw better conclusions of their patients/patient groups.

In summary, this study demonstrates that Z-scores calculated from the presently established reference standards perform well in a cohort of untreated ovarian tissue samples, thereby validating the model. The Z-score method allows quantification of genetic and cancer treatment effects on clinical fertility preservation material. Adoption of follicular density Z-scores is recommended for the estimation of normality of ovarian reserve in young patients attending fertility preservation programmes, and in prediction analyses of their future fertility potential. The authors believe that use of the Z-score system will generate an uniform standardized method that contributes to estimation of the quality of individual patient samples, which will improve physicians' counselling of patients and parents on their future fertility options.

DATA AVAILABILITY

Data will be made available on request.

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SUPPLEMENTARY MATERIALS

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