Z-scores for comparative analyses of spermatogonial numbers throughout human development

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Objective: To normalize age-dependent effects on standardized measures of spermatogonial quantity such as the number of spermatogonia per tubular cross-section (S/T) or fertility index.
Design: Published quantitative histologic data on human spermatogonial numbers were used to create Z-scores for reference means and tested on archived testicular tissue samples.
Setting: Retrospective cohort study.
Patient(s): The sample cohort comprised testicular samples from 24 boys with cancer diagnosis and 10 with Klinefelter syndrome, as part of the fertility preservation programs NORDFERTIL and Androprotect, as well as archived histologic samples from 35 prepubertal boys with acute lymphoblastic leukemia and 20 testicular biobank samples.
Intervention(s): None.
Main Outcome Measure(s): Z-score values for S/T and fertility index on the basis of morphology and germ cell-specific markers (MAGEA4 and/or DDX4) were calculated, and the impact of cancer therapy exposure and genetic disorders on Z-score values was evaluated.
Result(s): The Z-scores for S/T values in the nontreated samples (-2.08 ± 2.20, $n = 28$) and samples treated with nonalkylating agents (-1.90 ± 2.60, $n = 25$) were comparable within ± 3 standard deviations of the reference mean value but differed significantly from samples exposed to alkylating agents (-12.14 ± 9.20, $n = 22$) and from patients with Klinefelter syndrome (-11.56 ± 4.89, $n = 8$). The Z-scores for S/T were correlated with increasing cumulative exposure to alkylating agents ($r = -0.7020$).
Conclusion(s): The Z-score values for S/T allow for the quantification of genetic and cancer treatment-related effects on testicular tissue stored for fertility preservation, facilitating their use for patient counseling. (Fertil Steril® 2021;116:713-20. ©2021 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Klinefelter syndrome, male fertility preservation, spermatogonia, cancer, Z-score

The production of sperm after puberty relies on the existence and functionality, as well as integrity, of spermatogonia. Spermatogonial density in the human testis decreases slightly from birth to 3 years of age, followed by a gradual increase until the age of 7 years, after which the numbers remain stable until the age of 11 years (1). These physiological processes can be disturbed by genetic or endocrine disorders, as well as medical interventions, such as chemotherapy or irradiation, leading to partial or complete depletion of spermatogonia, including spermatogonial stem cells, during childhood (2–6).

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Current strategies to preserve the reproductive potential of prepubertal patients center on the cryopreservation of testicular tissue samples containing spermatogonial stem cells (7). Therefore, knowledge regarding spermatogonial cell quantity in these cryopreserved tissue samples would help to establish future personalized fertility restoration strategies. However, to identify the adverse effects of genetic conditions and toxic exposures on spermatogonial number, the impact of developmental variation needs to be controlled. The present project aimed to develop a simple, cohort-independent method for normalizing age-dependent differences in spermatogonial numbers in testis tissue samples. We reviewed published quantitative histologic studies on human spermatogonial numbers per round tubular cross-section (S/T), as well as the fertility index (FI), and calculated Z-scores for the reference mean values from birth to adulthood. We further evaluated the performance of the Z-scores using archived biobank and cryopreserved testicular tissue samples from the NORDFERTIL and Androprotect fertility preservation programs (4–6).

MATERIALS AND METHODS

Ethical Approval

Ethical approval for use of testicular tissue samples was obtained from the Ethics Board of Karolinska Institutet and the Regional Ethics Board in Stockholm (Dnr 2013-2129-31/3 and Dnr 2014-267-31/4), the Ethics Board of the University of Helsinki (426/13/03/03/2015, 192/13/03/03/2013) and Turku University Hospital (Dnr 1905-32/300/05), and the Ethics Committee of the Medical Faculty of Münster (no 2011-520-f-S).

Patient Cohorts and Controls

The NORDFERTIL sample cohort consisted of testicular samples from 12 boys (7.6 ± 3.8 years of age) with cancer diagnosis from Sweden, Finland, and Iceland who were enrolled in an experimental study involving testicular cryopreservation, as part of the NORDFERTIL research program as previously described (6). The Androprotect sample cohort consisted of testicular samples from 12 boys (11.8 ± 2.5 years of age) with cancer diagnosis without previous cancer therapy and from 10 boys (14.2 ± 1.7 years of age) with Klinefelter syndrome (4). Written and verbal information about the respective research projects was provided to the parents, and when appropriate to the patient, before informed consent was obtained in both research projects. The archived testicular material consisted of histologic samples from 35 prepubertal boys (8.3 ± 3.5 years of age) with acute lymphoblastic leukemia who had undergone routine testicular biopsy to examine possible testicular leukemia at the cessation of antileukemic therapy at University Central Hospitals in Helsinki and Turku between 1979 and 1995 as previously described (5). Alkylating agent exposure was calculated as mean cumulative cyclophosphamide equivalent doses (CEDs) (8), and anthracycline exposure was calculated as cumulative doxorubicin isotoxic dose equivalents using conversion factor 1 for doxorubicin and 0.833 for daunorubicin (Supplemental Table 1). Twenty testicular samples (4.5 ± 3.7 years of age) with unknown testicular pathology from the biobank of the Department of Pathology, Karolinska University Hospital, served as an additional control group. In further analysis, the nontreated group included samples from biobank controls and cancer patients without treatment. The nonalkylating and alkylating groups included samples from chemotherapy-treated patients from fertility preservation programs and archived testicular material (Supplemental Table 1).

Tissue Processing and Histologic Analyses

Testicular biopsy samples from the NORDFERTIL program and archived testicular material were fixed in either formalin or Bouin's solution. Biopsy samples from the Androprotect program were fixed in Bouin's solution. All samples were embedded in paraffin, sectioned (3–5 μm), and stained with hematoxylin and eosin or with periodic acid–Schiff as previously described (4, 6). To compare the methods to detect spermatogonia in nonconsecutive (distance >15 μm) fixed tissue sections from the same patient samples, immunostaining against two germ cell-specific markers (MAGEA4 [monoclonal mouse antibody, a donation from Prof. G.C. Spagnoli, University Hospital of Basel, CH] or mouse anti-MAGEA4 [ab139297, Abcam, Cambridge, UK; final concentration, 2.5 μg/mL] and mouse anti-DDX4 [ab13840, Abcam, Cambridge, UK; final concentration, 5 μg/mL] or rabbit anti-DDX4 [ab27591, Abcam, Cambridge, UK; final concentration, 5 μg/mL]) was performed as previously described (4, 6). For negative controls, primary antibodies were replaced by mouse immunoglobulin G (sc-2025, Santa Cruz Biotechnology, Dallas, Texas, USA; final concentration, 4 μg/mL; or ab27478, Abcam, Cambridge, UK; final concentration, 2 μg/mL) or rabbit immunoglobulin G (ab172730, Abcam, Cambridge, UK; final concentration, 8.9 μg/mL) diluted in blocking buffer (TBS/NDS/BSA). Spermatogonial numbers per round tubular cross-section (S/T) and the FI were determined in a blinded approach by an experienced examiner. The following formulas were used:

$$S/T = \frac{number\ of\ spermatogonia}{number\ of\ round\ tubules}, FI = \frac{number\ of\ round\ tubules\ with\ spermatogonia}{number\ of\ round\ tubules} * 100$$

Spermatogonia were identified on the basis of morphology (size, shape, and location) (9) in periodic acid–Schiff- and hematoxylin and eosin-stained sections and DDX4 and/or MAGE4 expression. At Karolinska Institutet, an Eclipse E800 microscope (Nikon; Japan) or an LMS700 confocal microscope (Zeiss; Germany) was employed for analysis. At the University of Münster, images of the entire tissue sections were captured with a PreciPoint M8 microscope and scanner (PreciPoint, Freising, DE) and subsequently analyzed using ViewPoint light (1.0.0. 9628, PreciPoint, Freising, DE). A minimum of 25 tubular cross-sections per

sample were evaluated to achieve validity of results (10) (Supplemental Table 1). Two samples from patients with Klinefelter syndrome and four from the nontreated group did not reach the criteria and were, therefore, excluded from analysis. The tubules were considered as round if the ratio of the longer to the smaller diameter of the tubule was ≤ 1.5.

Data Extraction for Reference Material and Calculation of Combined Means and Standard Deviations

Z-scores below or above the reference mean value (numerical value of standard deviations [SDs]) for S/T and FI were calculated. Reference values were on the basis of a previously published systematic literature search in PubMed focused on developmental and quantitative reports of spermatogonial numbers as determined by morphology (1). For age groups 0–10 years, all data sets that reported the mean and SD for S/T (Supplemental Table 2) (9, 11–13) and FI (Supplemental Table 3) (9, 13) in one age group were included. Pooled data was filtered out (10, 14–16). For the pubertal age group (10–14 years), multiple pooled data sets that fit the age limits were included (Supplemental Tables 2 and 3) (9, 11–13, 17). For adolescent and adult age groups, 14–18 and 25–40 years, respectively, the mean and SD values of only one published data set was identified (Supplemental Tables 2 and 3) (13).

In data sets where only the median and lower and upper limits were given, the mean and SD were estimated using the following equations described in the publication by Hozo et al. (18):

$$M = \frac{A + 2 * m + B}{4}$$

$$SD^2 = \frac{1}{12}\left(\frac{(A - 2 * m + B)^2}{4} + (B - A)^2\right)$$

where M = mean, m = median, A = lower limit, B = upper limit, and SD = standard deviation.

In situations where there were two data sets for the same age groups, the combined mean and SD for both data sets were calculated (Supplemental Tables 2 and 3) (19). If there were more than two data sets to combine, data sets were combined sequentially:

$$N_{12} = N_1 + N_2$$

$$M_{12} = \frac{N_1 * M_1 + N_2 * M_2}{N_1 + N_2}$$

$$SD_{12} = \sqrt{\frac{(N_1 - 1) * SD_1^2 + (N_2 - 1) * SD_2^2 + \frac{N_1 * N_2}{N_1 + N_2} * (M_1 - M_2)^2}{N_1 + N_2 - 1}}$$

where N = sample size, M = mean, and SD = standard deviation. The number in the subscript indicates the number of the data set: 1 = data set 1, 2 = data set 2, and 12 = combined data.

Z-score Calculation for Study Samples

Z-scores for study samples were calculated using the values of samples and combined means and SDs for respective age groups (Supplemental Tables 4 and 5):

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

where Z = Z-score, x = value of sample, M = combined mean, and SD = combined standard deviation.

All values in this equation must be normally distributed. We, therefore, first transformed the S/T values on a log-transformed scale using base-*e* log transformation described by Higgins et al. (20):

$$M_z = \ln(M) - \frac{1}{2} * \ln\left(\frac{SD^2}{M^2} + 1\right)$$

$$SD_z = \sqrt{\ln\left(\frac{SD^2}{M^2} + 1\right)}$$

where M = combined mean, SD = combined standard deviation, M_z = log-transformed combined mean, and SD_z = log-transformed combined standard deviation.

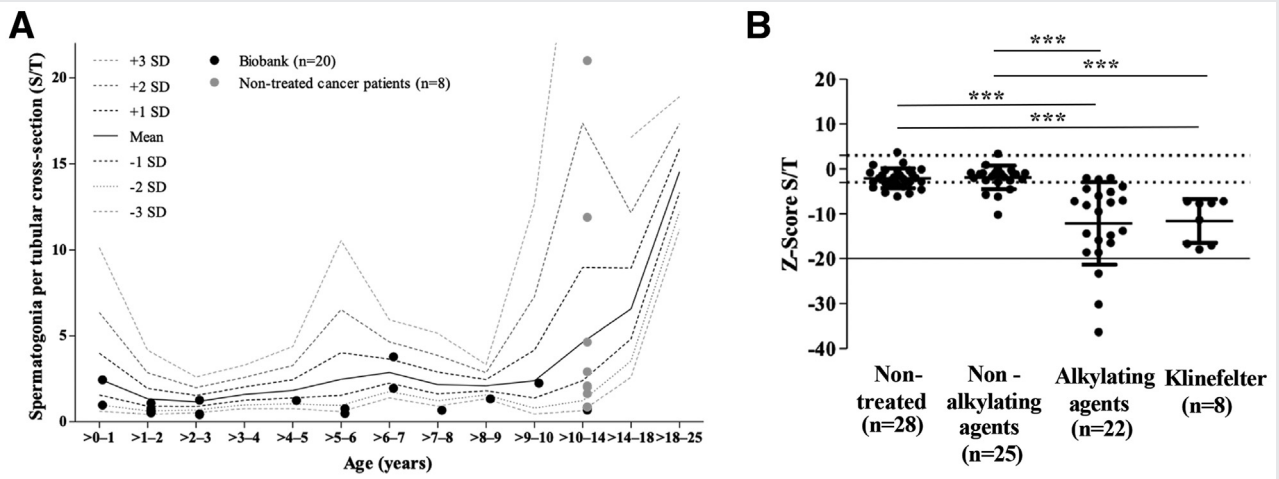
In the manuscript graphs, the log-transformed S/T values were transformed back to raw values by raising *e* to the power of the log-transformed value. Figure 1 shows how the Z-score for S/T values performs at 0, ±1, ±2, and ±3 SDs in different age groups (Supplemental Table 4). As Z-scores cannot be calculated for an S/T value of zero, the minimal Z-score is presented if no spermatogonia were found in the tissue.

Values for FI were considered to be normally distributed, and no log transformation was performed. Supplemental Figure 1 shows how the Z-score for FI performs at 0, ±1, ±2, and ±3 SDs in age groups between 0 and 18 years (Supplemental Table 5). The published FI of healthy adults (>18 years old) is 100% with no variation and, thus, cannot be demonstrated with Z-scores.

Statistical Analysis

The Mann–Whitney U test (non-normally distributed variables) and Pearson correlation coefficient (R^2) were used to determine the relationships between spermatogonial quantity, age, and treatment characteristics. To show correlation between quantification of morphological staining and staining with antibodies MAGEA4 and DDX4, the Spearman correlation coefficient and linear regression analysis was used. Receiver operating characteristic curves and area under the curve (AUC) were reported to illustrate the diagnostic value of S/T Z-scores to predict any exposure to alkylating agents and high exposure (CED > 4,000 mg/m^2) to alkylating agents (8). After dose group comparison, *P* values were corrected using a Bonferroni–Holm correction to avoid a multiple test problem. Analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

FIGURE 1



Performance of the S/T Z-scores for spermatogonial numbers in control and fertility preservation material. **(A)** Number of spermatogonia per round tubule (S/T) by age in the nontreated samples including biobank control samples and samples from cancer patients without therapy. Data are plotted on *dotted lines* corresponding to the Z-scores for the reference mean values. **(B)** S/T Z-score values for the nontreated samples and samples exposed to nonalkylating and alkylating chemotherapy and for samples from patients with Klinefelter syndrome. The *lower line* of the group indicates the 25th percentile and the *upper line* 75th percentile. The *middle cross line* indicates the mean value of the group. *Dotted lines* correspond to the Z-score value ±3 SD. *(*P*<.0001).

Funke. Z-score for spermatogonial numbers. Fertil Steril 2021.

RESULTS

Z-score for Spermatogonia Quantity in Samples from Patients with Klinefelter Syndrome, Nontreated Samples, and Samples Exposed to Cancer Therapy

The distributions of demographic and treatment variables of males included in these analyses are shown in Supplementary Table 1 and were published previously (4–6). The S/T and FI values were on the basis of morphological analysis. The mean Z-scores for S/T values in the nontreated samples, including those from biobank controls and cancer patients without treatment (−2.08 ± 2.20, n = 28), were within ±3 SD from the age-dependent reference mean value (Fig. 1A). There were no differences in the mean S/T Z-scores between the nontreated samples and samples exposed to nonalkylating agents (−2.08 ± 2.20 vs. −1.90 ± 2.60, $P = .3496$). In contrast, samples from cancer patients exposed to alkylating agents exhibited a lower mean S/T Z-score value (−12.14 ± 9.20, n = 22) when compared with the nontreated samples ($P<.0001$) or samples exposed to nonalkylating agents ($P<.0001$) (Fig. 1). The demographic distributions of the patients with Klinefelter syndrome included in these analyses are shown in Supplementary Table 1 and were published previously (4). The mean Z-scores for S/T in samples from patients with Klinefelter syndrome (−11.56 ± 4.89, n = 8) were significantly lower compared with those from samples from the control group (−2.08 ± 2.20, n = 28) ($P<.0001$) (Fig. 1). Decreasing S/T Z-score values were correlated with increasing cumulative exposure to alkylating agents (r = −0.7020, $P<.0001$) (Fig. 2A) but not to anthracyclines (r = −0.2829, $P = .0540$) or patient age (r = 0.07341, $P = .6239$). An S/T Z-score less than −3 showed good diagnostic value (AUC, 0.93; 95% Confidence interval, 0.86–0.99) with 86% sensitivity and 84% specificity when identifying cancer patients exposed to any alkylating chemotherapy (Fig. 2B). An S/T Z-score less than −7 identified patients exposed to a high dose of alkylating chemotherapy (CED ≥ 4,000 mg/m^2) (AUC, 0.96; 95% Confidence interval, 0.90–1.01) with 93% sensitivity and 91% specificity (Fig. 2C).

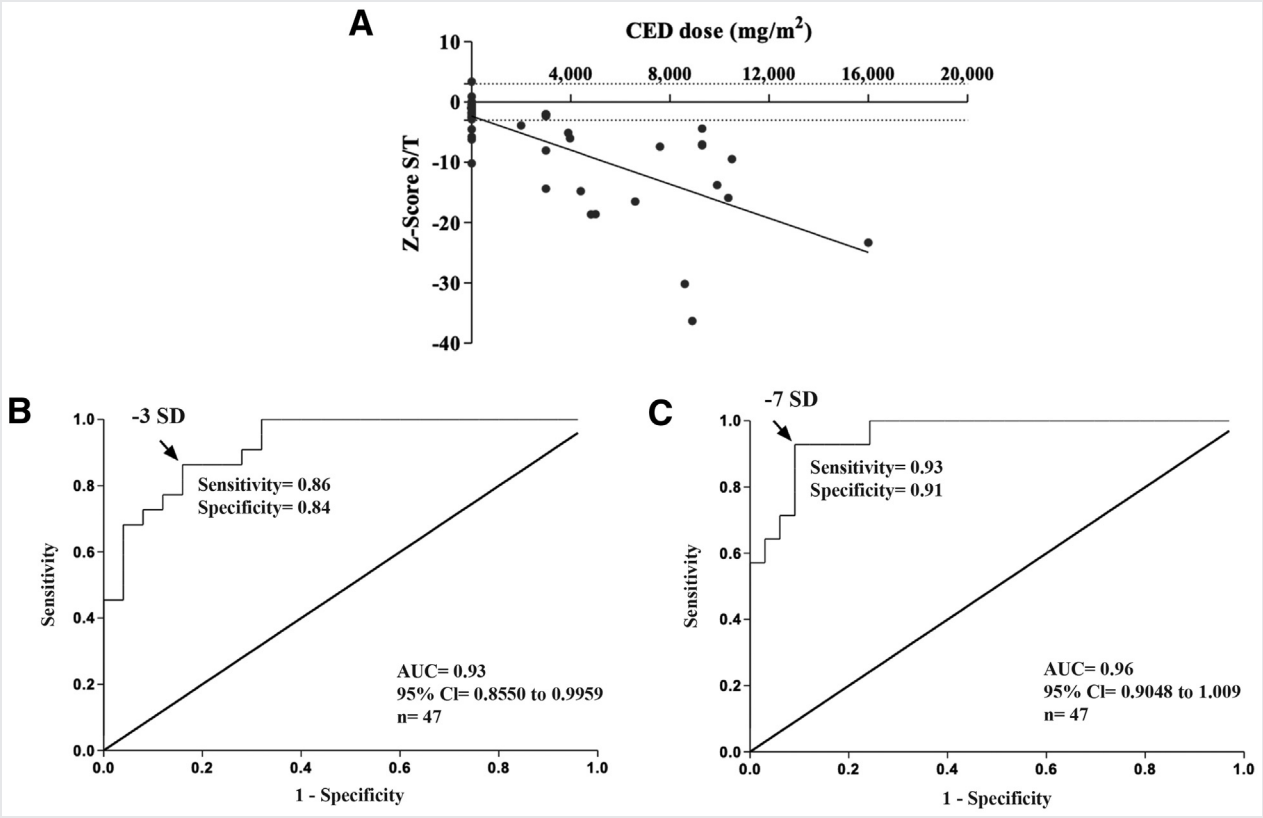
FI Z-score for Spermatogonia Quantity

The mean Z-scores for FI values in the nontreated samples showed high variation (−3.95 ± 3.57, n = 28) and were not within ±3 SD from the reference mean value (Supplementary Fig. 1A). The FI Z-scores of the nontreated samples (−3.95 ± 3.57) showed no difference to those of samples exposed to nonalkylating agents (−2.37 ± 3.90, $P = .0705$) but were significantly higher than the mean Z-scores of the samples exposed to alkylating agents (−10.41 ± 3.43, $P<.0001$) or samples from patients with Klinefelter syndrome (−12.13 ± 3.64, $P<.0001$).

Z-score and Methods to Detect Spermatogonia

A significant correlation existed in the mean Z-scores for S/T and FI values identified by morphology or by immunostaining with MAGE4 (r = 0.8424, $P<.0001$, Fig. 3; r = 0.8368, $P<.0001$, Supplemental Fig. 2, respectively) and DDX4

FIGURE 2



Age-dependent S/T Z-scores for spermatogonial numbers in quantification of cancer treatment effect. **(A)** Significant correlation (r = −0.7020, *P*=<.0001) between exposure to alkylating agents by cumulative cyclophosphamide equivalent doses (CEDs) (mg/m^2) and Z-score values for spermatogonial per round tubule (S/T). Receiver operating characteristic (ROC) curve for S/T Z-score detecting patients **(B)** without exposure to alkylating agents and **(C)** with exposure to high cumulative dose of alkylating agents (CED > 4,000 mg/m^2). The cutoff values and corresponding sensitivities and specificities are indicated by *arrows*.

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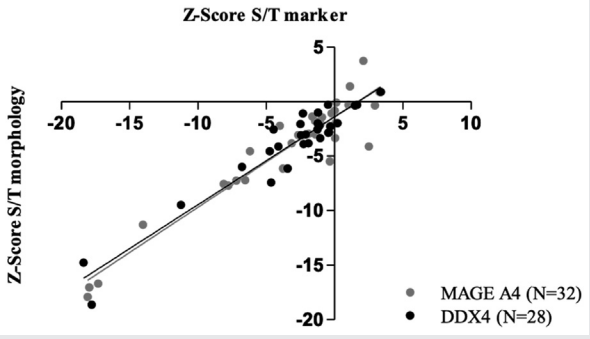
FIGURE 3



Methods to identify spermatogonia do not impact age-dependent S/T Z-scores for spermatogonial numbers. A significant correlation exists in Z-scores for spermatogonial numbers per tubular cross-section (S/T) between morphology-based and MAGE4 (r = 0.8424, *P*<.0001) and DDX4 (r = 0.8030, *P*<.0001) expression-based methods to identify spermatogonia. The study includes biobank control material (n = 20) and testicular samples from fertility preservations cohorts (n = 16).

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(r = 0.8030, *P*<.0001, Fig. 3; r = 0.8106, *P*<.0001, Supplemental Fig. 2, respectively). Curves were superimposable, indicating that the classification properties of the three measures were virtually identical. No difference in the mean Z-score values for S/T and FI were detected when the three different methods were compared in the nontreated tissue samples (morphology-based evaluation, −2.51 ± 1.83 and −4.85 ± 2.99, n = 15; MAGE4 expression-based evaluation, −0.92 ± 2.7 and −2.38 ± 4.76, n = 15; DDX4 expression-based evaluation, −1.34 ± 2.24 and −4.15 ± 3.67, n = 15, S/T and FI, respectively).

DISCUSSION

Fertility preservation makes use of cryopreserved testis tissue from a highly heterogeneous patient cohort in terms of differing age and developmental reproductive as well as varying diagnosis and therapy exposure. All of these factors can impact spermatogonial numbers, which can consequently influence the potential outcome of approaches aiming to restore fertility. We, therefore, developed Z-scores for spermatogonial numbers to normalize variation associated with testicular development.

We demonstrated that the Z-scores for S/T values for the nontreated samples were within the normal range (±3 SD, including 99.7% of the reference cases) from the mean and SD of the respective age groups, confirming the adequate performance of Z-scores. In addition, we showed that the Z-score values performed similarly when cancer patient samples were included from different hospitals and treatment cohorts and when morphology or immunohistochemical-based methods were used to detect spermatogonia. Our observations suggest that either morphology or immunohistochemical methods can be used for calculating Z-scores in clinical studies. A primary advantage of using Z-scores is that the analysis of patient samples is independent of age distribution in the study population. Quantification of spermatogonial numbers standardized by age will allow data from patients with similar genetic backgrounds or therapy exposures to be combined with larger study populations to enable meta-analyses to be performed.

We further identified a cutoff mean S/T Z-score of −7 that showed good diagnostic value when identifying cancer patients exposed to high doses of alkylating agents (>4,000 mg/m^2). Exposure to CED > 4,000 mg/m^2 has previously been reported to be associated with long-term impairment of spermatogenesis (8). We further identified a cutoff mean S/T Z-score of −3 within the normal range to distinguish nonalkylating treated cancer patients from alkylating treated patients. Our observations confirm the previous hypothesis that nonalkylating treated cancer patients can be used as reference group (6). The clinical conclusion of these findings is that S/T Z-scores > −3 reflect a spermatogonial quantity that can be regarded as normal, while S/T Z-scores < −7 reflect a significant depletion of spermatogonial numbers. In addition, the age-dependent Z-scores performed consistently among patients with Klinefelter syndrome. The mean S/T Z-score of −12 among patients with Klinefelter syndrome (12–17 years old) reflects the previously reported deterioration of spermatogenesis before adolescence (2).

Z-scores for the FI were heterogeneous within the different groups and mostly outside the normal range (±3 SD) of the reference values. One explanation could be that only a small part of the testis was examined and evaluation of 25 tubules may not be high enough to gain a complete overview of the tissue composition and high spatial variation in spermatogonial density. Significant longitudinal growth of seminiferous tubules takes place during postnatal primate testicular development and may further increase the variation in spermatogonial density within the tubules in pediatric testicular samples (21).

A limitation of this study is the reliability of reference material obtained from a small number of old quantitative histologic studies. In the five reported studies, most of samples were from autopsies, and several of the older subjects had undergone surgical orchiectomy. It is possible that this difference with regard to the source of the testicular samples influences our findings as a factor other than age, as our cohort consisted only of tissue samples obtained from biopsies. Furthermore, because age-grouping strategies were unified, we excluded four (10, 14–16) studies that contained pooled data for prepubertal ages, which further decreased the data included. This in particular concerns the FI data, where reference material was even more limited compared with S/T data. Calculated values (S/T and FI) for age groups 14-18 and adults were on the basis of only one data set. The tissue-processing methodologies referenced in the selected studies included histologic spermatogonial quantity assessments without correction for tissue shrinking. These methods are still routinely used in clinical and research settings (1, 22, 23), and the number of evaluated tubular cross-sections in the selected studies was in a range recommended to achieve result validity (1, 10).

In summary, we have demonstrated that the Z-score values for S/T perform adequately in nontreated testicular tissue samples and allow quantification of genetic and cancer treatment effects on clinical fertility preservation material. We recommend the adoption of the S/T Z-scores for the reference mean value for the estimation of spermatogonial quantity in fertility preservation programs and in future prediction analyses. The use of the Z-scores will create a uniform standardized method to estimate the quality of the individual patient sample and improve physicians' counseling of patients and parents on boys' future fertility options.

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Puntaje Z para análisis comparativos de números de espermatogonias durante el desarrollo humano.

Objetivo: Normalizar los efectos dependientes de la edad sobre las medidas estandarizadas de cantidad de espermatogonias, tales como el número de espermatogonias por sección tubular (S/T) o el índice de fertilidad.

Diseño: Se utilizaron publicaciones de datos histológicos cuantitativos de números de espermatogonias humanas para crear puntajes Z para promedios de referencia y se probaron en muestras testiculares almacenadas.

Marco: Estudio retrospectivo de cohorte.

Paciente(s): La cohorte de la muestra se compuso de muestras testiculares de 24 niños con diagnóstico de cáncer y 10 con síndrome de Klinefelter, como parte de los estudios de preservación de la fertilidad NORDFERTIL y Androprotect, así como muestras histológicas archivadas de 35 niños pre-púberes con leucemia linfoide aguda y 20 muestras testiculares de un banco biológico.

Intervención(es): Ninguna.

Medida(s) principal(es) de resultado(s): Se calcularon los valores de puntaje Z para S/T e índice de fertilidad basado en morfología y marcadores específicos de células germinales (MAGEA4 y/o DDX4) y se evaluó el impacto de la exposición al tratamiento oncológico y de las enfermedades genéticas sobre los puntajes Z.

Resultado(s): Los puntajes Z para los valores de S/T en las muestras no tratadas (-2,08 $\pm$ 2,20, n = 28) y muestras tratadas con agentes no alquilantes (-1,90 $\pm$ 2,60, n = 25) fueron comparables dentro de $\pm$3 desviaciones estándar del valor medio de referencia, pero difirieron significativamente de las muestras expuestas a agentes alquilantes (-12,14 $\pm$ 9,20, n = 22) y de los pacientes con síndrome de Klinefelter (-11,56 $\pm$ 4,89, n = 8). Los puntajes Z de S/T se correlacionaron con una exposición incremental a agentes alquilantes (r = -0,7020).

Conclusión(es): Los valores de puntaje Z de S/T permiten cuantificar los efectos genéticos y los relacionados con tratamiento oncológico sobre el tejido testicular almacenado para preservación de la fertilidad, permitiendo su uso para el consejo del paciente.